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The Activity of Streptomycin in the Presence of Serum and Whole Blood.*

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(Introduced by P. H. Long.)

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Previous studies[1] have shown that streptomycin resistant variants of Staphylococcus, Pneumococcus and Streptococcus grow as well in a bactericidal test as their parent susceptible strains. It was presumed that, in the presence of streptomycin, the additive effect of fresh whole blood could be demonstrated on both the susceptible and resistant strains. The effect on the latter, it was felt, would be minimal, but such investigation was of importance because of the numerous examples of streptomycin resistance which had been observed to develop during the course of therapy. No studies have been reported in which the susceptibility of the resistant organism was tested by the bactericidal technique in the presence of added streptomycin—a condition which would naturally occur in a patient under therapy.

The following studies were made to investigate the inter-relationships which may be present when bactericidal tests are performed with a strain of *Staphylococcus aureus* in the presence of streptomycin. In addition, the various important known components of the bactericidal test such as phagocytosis, hemolysis of red blood cells, immune serum and labile constitutents were investigated. All

* These investigations were supported by grants from Abbott Laboratories, Eli Lilly and Company, Lederle Laboratories, Inc., Parke Davis and Company, and the Upjohn Company.

1 Chandler, C. A., and Schoenbach, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 208.

these factors were controlled, wherever possible, by simultaneous duplicate observations in which the sensitive parent strain of the staphylococcus was used.

Materials and Methods. The strain used throughout the tests was a *Staphylococcus aureus* (Merck test strain SM) obtained from Sydenham Hospital. The strain of staphylococcus resistant to 1000 μg of streptomycin was derived from the parent strain as previously described.[1] Following initial experiments all cultures were transferred at intervals of 3 to 4 weeks on suitable culture media not containing streptomycin. No attempt was made to maintain the original virulence of the strains during the experimental period.

The susceptibility of the strains to streptomycin was determined by the use of a series of tubes of nutrient broth (PDW)† containing streptomycin in appropriate concentrations. Each tube, within the range, contained 10% less streptomycin as measured against the initial tube containing the most streptomycin in a regular progression, so that the differences from tube to tube were uniform. Each tube was inoculated with 0.05 cc of an 18-hour broth culture in such dilution as to yield an inoculum of about 200,000 organisms per one cc of medium. The tubes were incubated at 37°C and readings were made at the end of 24 hours.

The bactericidal power of freshly defibrinated human blood on the staphylococcus was determined in the following manner. Five hundredths cc of each 10-fold serial dilution (10^{-1} to 10^{-6}) of an 18-hour broth culture was added to 0.25 cc of freshly defibrinated human blood in each of 6 sterile pyrex tubes. These tubes were sealed, placed in a rotating box and maintained at 37°C for 24 hours. After preliminary observation, incubation without rotation was continued for another 24 hours, after which the tubes were opened and the contents cultured on blood agar plates. In order to determine the number of organisms added to each tube of blood, count plates were made of the 10^{-5} and 10^{-6} dilutions. This technique was varied as described in each group of experiments by the substitution of serum as a substrate and by the addition of immune serum, streptomycin, etc.

Phagocytic activity was measured in the following manner: 0.25 cc of whole, defibrinated blood was measured into sterile pyrex tubes. To the above tubes, 0.1 cc serum, either normal or immune was added. Streptomycin, in a final concentration of 500 μg was then introduced into some of the tubes. Fluid volumes in all tubes were made equal by the addition of appropriate amounts of broth. Finally each tube was inoculated with 0.05 cc of a 3-hour culture of either the susceptible or resistant strain. The tubes were then sealed in an oxygen flame, slowly rotated for 30 minutes at 37°C, broken open, and one drop of the contents of each was smeared on a slide as a blood film. The slides were then stained with Wright's stain and examined with the oil immersion lense. A count was made of the number of intracellular staphylococci contained in 25 polymorphonuclear leukocytes and the percentage of cells taking part in the phagocytosis was noted.

Growth curves were obtained by making bacterial counts at 2, 4, 6 and 24 hours. The number of organisms was estimated by making count plates of suitable dilutions of the culture. Estimation of turbidity in a photoelectric colorimeter with readings at hourly intervals was used in each instance to confirm these counts.

Immune rabbit serum was obtained in the following manner. A white albino rabbit was injected intravenously with a washed, heat-killed suspension of organisms. The suspension was prepared from an 18-hour broth culture of the susceptible parent strain of the staphylococcus. The rabbit received injections 4 times a week for 3 weeks. During the final week living organisms were used. The final agglutinative titre of the serum prepared as described above was 1-20 against both the susceptible strain and the resistant strain. Thread agglutination was observed when both these strains were grown in media containing this immune serum.

One lot (Winthrop-Pfizer Lot No. P4713) of streptomycin was employed throughout this study. It had a labeled potency of 1000

† Peptone-Dextrose-Water.

TABLE I.
Bactericidal Studies.

Strain	Substrate	Minimum No. of organisms required to initiate growth per cc of blood or serum				
		Streptomycin, 0 μg	Streptomycin, 5 μg × million	Streptomycin, 10 μg × million	Normal rabbit serum	Immune rabbit serum
Parent susceptible	Whole blood	420	4.2	42	640	640
	Serum	520*	52.0*	52*	—	—

* Human serum.

TABLE II.
Proportion of Organisms in Resistant Staphylococcus Culture Growing at Indicated Streptomycin Concentrations.

Concentration of streptomycin in medium (μg per cc)							
0 colonies		500 colonies		750 colonies		1000 colonies	
No.	%	No.	%	No.	%	No.	%
700	100	700	100	600	85.7	600	85.7

units per milligram of pure base.

Results. (1) The sensitivity to streptomycin of the susceptible strain was re-evaluated in the light of previous reports that serum or whole blood inhibited the action of streptomycin.[2,3,4] In broth, the bacteriostatic range of streptomycin for the susceptible strain was 5 to 12 micrograms on repeated determinations. In the presence of 60% fresh human or rabbit serum (final concentration), this same strain was inhibited by 5 μg of the antibiotic when the inocula varied from 520 organisms to 5.2 million per cc. Growth occurred in the presence of 5 and 10 μg of streptomycin when a large inoculum was used (52,000,000 organisms).

In fresh whole human blood, inhibition occurred in a concentration of 5 μg with inocula ranging from 420 to 4.2 million. With 10 μg of streptomycin present, an inoculum of 42 million organisms was necessary to produce growth. In both the serum and whole blood assays, these findings were confirmed by sub-culture after 48 hours. This was deemed necessary to rule out erroneous interpretations which may arise because of nonspecific turbidity or hemolysis.

In summary, as shown in Table I, the susceptibility of this strain of staphylococcus to streptomycin was not affected by the presence of serum or whole blood within the limits of measurement which were employed. In addition, there was no real indication that the bacteriostasis achieved with streptomycin was augmented by the bactericidal activity of whole blood. In the absence of streptomycin, there was no indication that this strain of staphylococcus was affected by normal whole blood or when immune serum was added to the system.

(2) The resistant strain of staphylococcus had been derived in this laboratory from the parent strain and had been found to grow readily in broth containing more than 1000 μg of streptomycin. Colony counts performed by seeding agar plates containing either no streptomycin or up to 1000 μg of streptomycin revealed no essential difference in the number or type of colonies. This is shown in Table II. In the bactericidal test, as shown in Table III, the resistant strain was not inhibited by normal human blood or when immune serum was added to the system. Thus in all respects, except for its resistance to streptomycin, it resembled the parent strain.

When the resistant strain was grown in the

2 May, J. R., Voureka, A. E., and Fleming, A., *Brit. Med. J.*, 1947, **1**, 627.

3 Henry, R. J., Berkman, S., and Housewright, R. D., *J. Pharm. and Exp. Therap.*, 1947, **89**, 42.

4 Hobby, G. L., and Lenert, T. F., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 242.

TABLE III.
Bactericidal Studies.

Strain	Substrate	Minimum No. of organisms required to initiate growth per cc of blood or serum						
		Streptomycin, 0 μg	Streptomycin, 125 μg	Streptomycin, 500-1000 μg	Normal rabbit serum	Immune rabbit serum	Immune rabbit serum + 125 μg*	Immune rabbit serum + 500-1000 μg*
Resistant	Whole blood	320	320	320	340	340	340	340
	Serum	360†	360†	360†	280‡	280‡	280‡	280‡

* Streptomycin.
† Human serum.
‡ Rabbit serum.

TABLE IV.
Bactericidal Studies.

Strain	Duration of growth, hr	Substrate	Minimum No. of organisms required to initiate growth per cc of blood or serum					
			Normal rabbit serum	Normal rabbit serum + 125 μg*	Normal rabbit serum + 500 μg*	Immune rabbit serum	Immune rabbit serum + 125 μg*	Immune rabbit serum + 500 μg*
Resistant	24	Whole blood	3600	3,600,000	360,000	360	360,000	3,600,000
	48	" "	340	—	—	340	340	340
Resistant	24	Rabbit serum	280	2800	280,000	280	280	28,000
	48	" "	280	280	2800	280	280	280

* Streptomycin.

TABLE V.
Bactericidal Studies.

Strain	Time inoculated after mixing	Substrate	Minimum No. of organisms required to initiate growth per cc of serum					
			Normal rabbit serum	Normal rabbit serum + 125 μg*	Normal rabbit serum + 500 μg*	Immune rabbit serum	Immune rabbit serum + 125 μg*	Immune rabbit serum + 500 μg*
Resistant	0	Rabbit serum	280	280	2800	280	280	280
	24 hr	Rabbit serum	160	1600	1600	160	160	16,000

* Streptomycin.

TABLE VI.
Phagocytic Studies.

Strain	Normal rabbit serum		Immune rabbit serum		500 μg streptomycin		Normal rabbit serum + 500 μg streptomycin		Immune rabbit serum + 500 μg streptomycin	
	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci
Parent susceptible	204	68%	625	94%	126	40%	—	—	—	—
Resistant	960	92%	1100	92%	832	72%	997	84%	775	72%

presence of 60% rabbit serum containing streptomycin, definite inhibition of growth was observed. With as little as 125 μg of streptomycin per cc, this strain, resistant to 1000 μg per cc, showed retardation in growth after 24 hours when small inocula (200 to 300,000 organisms per cc) were used. At the end of 48 hours, however, growth equal to that in the controls was observed. A similar pattern of growth inhibition with streptomycin was noted in the media containing whole blood with or without added immune serum. Inhibition was somewhat more apparent in these latter experiments because hemolysis was delayed. These results are summarized in Table IV.

An investigation was then undertaken into the cause of this transient inhibition of growth. That streptomycin might be inactivated or bound by one of the serum constituents, was considered a distinct possibility. If so, this would account for the subsequent escape from the inhibitive effects of streptomycin. Accordingly, duplicate experiments were devised in which normal rabbit serum which had been inactivated by heat, with and without added immune rabbit serum, and to which streptomycin had been added in concentrations of 125 and 500 μg per cc was inoculated with organisms at 2 different times. One set was inoculated immediately and incubated for 24 hours while the duplicate set was incubated for 24 hours after which it was inoculated with organisms and again incubated for 24 hours. As shown in Table V, no difference was observed between the set inoculated immediately and that inoculated after 24 hours and, therefore, the escape from inhibitive effects of streptomycin after 24 hours could not be attributed to inactivation of streptomycin.

The lag observed when the resistant strain was grown in the presence of streptomycin cannot be attributed to the non-homogeneous distribution of streptomycin sensitive organisms as suggested by Berkman *et al.*[5] As shown in Table II, almost all organisms were able to grow at higher streptomycin levels

[5] Berkman, S., Henry, R. J., and Riley, D. H., *J. Bact.*, 1947, **53**, 567.

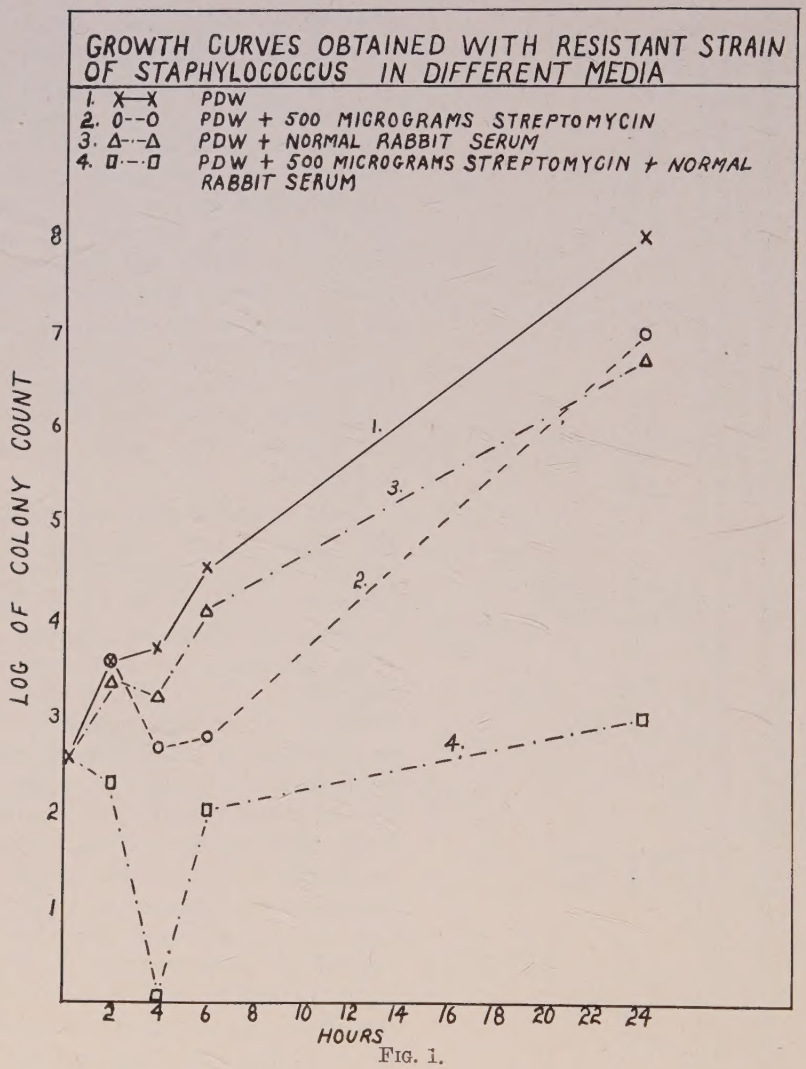

FIG. 1.

than were necessary to demonstrate this lag.

These observations are not necessarily in conflict with Price *et al.*[6] and Berkman and his co-workers[5] who found a marked difference in sensitivity to streptomycin when assayed in a somewhat deficient as compared with a highly nutritive medium. It would appear, however, from our observations that beyond a certain point progressive enrichment, although promoting growth of organisms, does not affect the final streptomycin titre.

[6] Price, C. W., Randall, W. A., Chandler, V. L., and Reedy, R. J., *J. Bact.*, 1947, **53**, 481.

Another possibility which was entertained was that streptomycin had stimulated phagocytosis and that, with the death of the leukocytes after 24 hours, growth had reappeared.

As shown in Table VI, it was found that in the presence of streptomycin, the phagocytic activity of the white blood cells was depressed in every instance and, therefore, the initial bacteriostasis could not be explained on the basis of enhanced phagocytosis at these streptomycin levels.

In the light of the above results, it seems probable that the only explanation of the in-

hibition of growth in the first 24-hour period in the presence of streptomycin, was a retardation in the rate of growth. Despite the observation that growth of the resistant strain in the absence of streptomycin did not differ materially from the susceptible strain in media containing broth or serum, this strain of staphylococcus, resistant to more than 1000 μg of streptomycin per cc, was markedly inhibited in its rate of growth by much lower concentrations of the antibiotic in the first 24-hour period. This inhibitory activity could be demonstrated with as little as 125 μg of streptomycin per cc. It was augmented when serum was present in the medium. Sample growth curves in streptomycin and non-streptomycin containing media are presented in Fig. 1. This type of growth curve resembles that noted with the sulfonamides which is apparent in the early phase of growth when small inocula are used.

Discussion. With the susceptible parent strain of staphylococcus, the addition of fresh whole blood to streptomycin did not augment streptomycin activity. On the other hand, the efficiency of the streptomycin was apparently not affected by the presence of blood and its constituents. It was, therefore, difficult to believe that the streptomycin was bound to some protein constituent in the serum unless the resultant combination retained the antibacterial properties of the antibiotic.

Inhibition of the rate of growth of a highly resistant strain in the presence of streptomycin was observed during the first 24 hours of incubation when the concentration of the latter was far below 1000 μg per cc. An explanation of this observation may be that the nutritional requirements of the organism had been modified with the acquisition of streptomycin resistance. The resistant strain appeared to grow as readily as the parent susceptible strain in the usual nutrient media, in the presence of serum and even in fresh whole human blood. When streptomycin was present in these media, however, the delay in the rate of growth of the resistant strain seemed to indicate that some nutritional factor or necessary enzymatic process had been materially affected. Eventually the resistant organism escaped from this streptomycin inhibition. The nature of this reaction may be of prime importance for the elucidation of the mode of action of streptomycin on microorganisms and possibly also the nature of the escape mechanism associated with resistance. These investigations are being continued at present.

These data may indicate that under normal conditions 2 alternative growth mechanisms are available to the normal organism. A general discussion of such mechanisms has been reviewed by Dubos.[7] In this study it would appear that one metabolic or synthetic pathway is blocked by streptomycin and another is insufficiently developed to permit normal growth. With the development of resistance, the second mechanism is augmented but apparently is still insufficient for optimal growth when streptomycin is blocking the first mechanism.

It is possible that the inhibitory activity of streptomycin is related to its combination with desoxyribonucleic acid in the bacterial cell.[8] Nucleoprotein metabolism is important in the growth and reproduction of bacteria. The combination of nucleic acid with streptomycin may interfere with the synthesis or utilization of nucleoproteins. Studies on the mode of action of penicillin have demonstrated that with *Staphylococcus aureus* dissimilation of ribonucleic acid was inhibited without any effect upon glucose oxidation.[9] The reaction inhibited by penicillin is probably the dissimilation of a pentose.[10] It has been claimed that streptomycin would also exhibit an inhibitory effect on the same reaction.[10]

When a strain resistant to streptomycin is isolated from a patient, it may be inferred that despite its ability to grow in high concentrations of the antibiotic *in vitro*, its rate of

[7] Dubos, R. J., *The Bacterial Cell*, 1945, Harvard University Press, Chapter VIII, Bacteriostatic and Bactericidal Agents, 275.

[8] Cohen, S. S., *J. Biol. Chem.*, 1947, **168**, 511.

[9] Krampitz, L. O., and Werkman, C. H., *Arch. Biochem.*, 1947, **12**, 57.

[10] Krampitz, L. O., Green, M. N., and Werkman, C. H., *J. Bact.*, 1947, **53**, 378.

growth in the body may be retarded by even small concentrations of the antibiotic. One should expect, therefore, that such resistant strains would have difficulty in maintaining themselves due to phagocytic mechanisms available *in vivo*. The impairment of phagocytosis by streptomycin should be studied in greater detail so that this important cellular factor in immunity may be more clearly defined.

Summary 1. The activity of streptomycin on a susceptible strain of *Staphylococcus aureus* was not changed in the presence of serum or whole blood. 2. The addition of fresh whole blood with or without immune serum did not augment streptomycin activity. 3. A resistant strain of *Staphylococcus aureus* was inhibited during the first 24 hours by streptomycin in low concentrations. This effect was augmented when serum was added to the medium. 4. Direct growth curves indicate that this inhibition by streptomycin of a resistant strain was attributable to interference with growth of the organism rather than inactivation of labile constituents. 5. Phagocytosis in the presence of high concentrations of streptomycin appears to be impaired. 6. The bacteriostatic activity of streptomycin was manifest on both susceptible and resistant strains. With the latter, this inhibition was transitory. The bacteriostatic mechanism and bactericidal mechanism are dissociated phenomena.

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Culture Procedures in Microbiologic Assays with *L. arabinosus* and *L. casei.*

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In the application of microbiological assay procedures, there have arisen questions relative to possible differences in the response of the test organisms as a result of variations in handling the stock cultures. Several investigators have mentioned the possible influence of stock culture media and conditions to which it is subjected on the subsequent response of an organism under assay environment, but few have made studies to determine the nature and extent of these influences. The most detailed work which has come to our attention has been the culture studies on *L. arabinosus* and *L. casei* by Nymon and Gortner.[1]

The present report is concerned with some notes made on frequency of transfer of stock cultures, preparation and dilution of inoculum and temperature and time of incubation of the assay tubes as these factors affect the assay.

Observations were made for a period of 6 months on the effect of frequency of transferring stock cultures of *L. arabinosus* on the subsequent growth response of this organism to graded amounts of nicotinic acid in the U.S.P.[2] assay medium. From a stab culture of *L. arabinosus* carried in agar medium (agar 1.5%, dextrose 1% and yeast 1%) 5 transfers were initially made into fresh agar medium of the same composition. After incubation at 30°C for 24 hours these cultures were stored in a refrigerator. One, designated "original", was maintained in the original stab without further transfer. The others were transferred at intervals of one, 2, 3, and 4 weeks respectively throughout the experiment. After incubation of the transplant for 24 hours it was returned to the refrigerator

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1 Nymon, M. C., and Gortner, W. A., *J. B. C.*, 1946, **163**, 277.

2 First U.S.P., XII Bound Supplement, 1943, 76.

TABLE I.
Effect of Frequency of Transfer of *L. arabinosus* on Titration Values in Nicotinic Acid Determinations After 24 Weeks.

Micrograms standard nicotinic acid	Titration values in cc 0.1N NaOH				
	0.1	0.2	0.3	0.4	0.5
Original culture	3.3	4.8	6.4	7.4	8.3
Culture transferred weekly	3.3	5.1	6.9	8.4	9.0
" " 2-week intervals	3.3	5.1	7.1	8.2	8.7
" " 3- " "	3.4	5.2	7.2	8.3	8.6
" " 4- " "	3.4	5.1	7.1	7.9	8.6

until the next transfer period. Periodic comparisons of microbiological standard response curves obtained with each of these cultures showed that there was no difference after 20 weeks. Only at the end of 24 weeks was there evidence that the "original" culture gave titration values differing from those obtained with the other 4 cultures. Results are given in Table I. Infrequent transfer over a longer period might eventually produce adverse effects, but these experiments indicate a remarkable stability of the *L. arabinosus* culture used.

The results of experiments to determine whether differences in incubation temperatures of the assay tubes caused any change in the behavior of *L. arabinosus* are presented in Table II. Two series of tubes incubated at 34°C and 35°C respectively showed identical titration values after 3 days. Two series of assay tubes incubated for periods of one, 2, and 3 days at 34°C and 37°C respectively, gave practically the same titration values after the first and after the second day but gave slightly higher values in the 34°C series after 3 days. In a third experiment incubation temperatures were held at 30°C and 33°C for periods of one to 6 days inclusively with no significant differences observed between any of the paired members. Titration values increased with increased period of incubation reaching a peak on the fifth day and remaining the same through the sixth. Duplicate titrations, the averages of which are given in the table, were more uniform after 3 days incubation than at the end of a shorter period.

TABLE II.
Effect of Temperature and Time of Incubation of *L. arabinosus* on the Titration Values in Nicotinic Acid Determinations.

Exp. No.	Temp. of incubation, °C	Time of incubation, days	Titration	
			Blank	Maximum
			cc 0.1N NaOH	
1.	34	3	1.4	9.6
	35	3	1.4	9.7
2.	34	1	0.6	5.7
	37	1	0.6	5.7
	34	2	0.7	8.3
	37	2	0.7	8.3
	34	3	0.8	9.1
	37	3	0.8	8.4
3.	30	1	1.0	6.1
	33	1	1.1	6.1
	30	2	1.2	8.3
	33	2	1.2	8.2
	30	3	1.3	9.3
	33	3	1.2	9.3
	30	4	1.2	10.4
	33	4	1.3	10.2
	30	5	1.2	11.2
	33	5	1.3	11.4
	30	6	1.3	11.1
	33	6	1.4	11.1

Inoculum is usually prepared from a broth culture after incubation for 20-24 hours in the basal medium used in the assay. It has been found, in the case of *L. arabinosus*, that subsequent storage of the incubated culture in a refrigerator for an additional 24 hours before preparation of the inoculum has no effect upon the assay results.

L. casei is known to be more susceptible to environmental influences than *L. arabinosus*. In Table III are data showing the effects of age and concentration of inocula of *L. casei* on the response of this organism to graded amounts of folic acid. A modification of the U.S.P. niacin medium[2] was used for these comparisons. Inocula were prepared from broth cultures incubated at 30°C for 16, 24, 40, and 48 hours. In addition there were included 16 hour broth cultures to which had been added a preparation containing strep-

TABLE III.
Effect of Age of Culture and Concentration of Inoculum on Titration values in Folic Acid Determination Using *L. casei*.

Micrograms standard folic acid	0	.0002	.0004	.0006	.0008	.001
	cc 0.1N NaOH					
16 hr culture—undil.	3.4	5.5	6.9	8.3	9.3	10.5
16 " " (containing strepogenin)—undil.	3.1	5.0	6.5	7.9	9.1	10.4
16 " " " " "	3.1	5.1	6.5	7.8	9.1	10.2
24 " " undil.	3.3	5.2	6.7	8.1	9.4	10.5
24 " " dil.	3.1	5.1	6.5	7.8	8.8	10.1
40 " " undil.	1.2	1.3	1.4	1.6	1.8	1.85
40 " " dil.	2.8	4.5	5.9	6.9	7.8	8.7
48 " " undil.	1.4	1.9	2.4	2.9	3.2	3.5
48 " " dil.	2.4	4.2	5.2	6.2	7.0	7.8

ogenin that was found to be active in accelerating the initial growth of *L. casei*, on purified media as originally reported by Sprince and Woolley.[3] An undiluted portion of each inoculum was tested as well as a portion diluted to the same turbidity as the 16 hour preparation without the strepogenin. There was little difference in the response to graded amounts of folic acid shown by inocula prepared from the 16 hour culture, with or without strepogenin, and the 24 hour culture. Dilution of the inoculum from either the 16 hour culture with strepogenin or the 24 hour culture did not alter the degree of this response. The concentrated inocula from the 40 and 48 hour cultures gave growth in all of the tubes insignificantly above that in the blanks. The diluted inocula for these 40 and 48 hour cultures gave a usable growth range but one that was considerably less than that produced by inocula from the younger cultures. It appears that the best results with *L. casei* can be obtained by preparing inocula from cultures not more than 24 hours old.

Discussion. Failure of the frequency of transfer of our stock culture of *L. arabinosus* to affect the nicotinic acid assay results does not agree with the findings of Nymon and Gortner. These investigators reported a gradually decreased linearity of response in their culture of *L. arabinosus* transferred every 3 or 4 weeks in yeast extract-glucose-agar medium with incubation at 37°C. This response was improved by culturing in enriched media, reducing the incubation temperature to 30°C, the optimum reported by Bergey *et al.*[4] for *L. arabinosus*, and increasing the frequency of transfer. They did not include a consideration of incubation temperatures of the assay tubes in their report.

Price and Graves[5] found, in the determination of riboflavin by *L. casei*, that incubation temperature variations of 4-5°C from the optimum of 40-42° for this organism resulted in a decrease of 25% in the titration values. In the present study, no such striking differences in the behavior of *L. arabinosus* were observed with temperature variations in the incubation of assay tubes in the nicotinic acid determination. However, it is well recognized that *L. casei* is more readily affected by environmental conditions than *L. arabinosus*. Following earlier recommendations of Snell and Strong[6] for the preparation of inocula, Bird *et al.*[7] obtained more satisfactory results when they used diluted inocula. This agrees with our findings for inocula made from older cultures of *L. casei*. On the other hand no difference could be seen in undiluted and diluted inocula from cultures not more than 24 hours old. Inocula from young cultures were superior to inocula from old cultures even though the latter had been diluted.

[3] Sprince, H., and Woolley, D. W., *J. Exp. Med.*, 1944, **80**, 213.

[4] Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P., *Bergey's Manual of Determinative Bacteriology*, 1939, Baltimore, 5th edition.

[5] Price, S. A., and Graves, H. C. H., *Nature*, 1944, **153**, 461.

[6] Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, **11**, 346.

[7] Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D., *J. B. C.*, 1945, **159**, 631.

Summary. The results indicate that the culture of *L. arabinosus* used in these studies for microbiological assay was not easily affected by frequency of transfer of stock cultures or by incubation of assay tubes at fixed temperatures between 30°C and 37°C. In the case of the culture of *L. casei,* both the dilution of the inoculum and the age of the culture from which it was prepared affected assay titration values.

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Recovery of Virus from Throats of Poliomyelitis Patients.*

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In discussions of the epidemiology of poliomyelitis much importance has been placed on the presence or absence of virus in the nose and throat of patients and of associates in so far as such findings might support theories of transmission of the disease by means of droplets. A review of literature on the subject in 1938[1] indicated that virus was reported in the nasopharynx, tonsils or trachea of patients in 15% of 105 examinations made during the first 5 days of illness and in 7% of 182 made later. Conflicting evidence has been presented during the last decade. Sabin and Ward found virus in the pharyngeal wall of 4 of 7 patients dead within 6 days of onset,[2] and Kessel *et al.* reported positive results with tonsil-adenoid tissue in 3 of 6 autopsy specimens.[3] Howe and Bodian detected virus in pooled throat swabs from at least 2 of 28 healthy children and from 1 of 6 juvenile, familial associates of cases; the positive associate may have been a nonparalytic case since the child had fever and diarrhea at the time of collection of the specimen.[4] Oropharyngeal swabs obtained by the same authors, within 3 days after onset of illness from 10 of 23 patients yielded virus, but none of 13 swabs taken between the 4th and 9th days was positive.[5] In a similar study by Horstmann, Melnick and Wenner, only one of 19 swabs and one of 15 washings obtained during the first week of illness contained virus;[6] the specimens from 10 of the 19 patients in the study were obtained within 3 days after onset of symptoms. Kramer, Hoskwith and Grossman[7] tested 18 washings of the nasopharynx and recovered virus from 2 which had been collected 5 and 9 days after onset, respectively. Ward and Walters tested material from the nose and throat of 19 patients between the second and fourth days of illness. Virus was isolated from cloth masks over the nose and mouth of two patients, from a nasal swab of one of these, from pharyngeal swabs of the second and from 5 others.[8]

This report describes attempts to recover virus from pharyngeal washings of a group of poliomyelitis patients during the outbreak

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TABLE I.

Dallas Throat Washings.																	
Day after onset of symptoms specimen was collected	3	4	6	7	7	7	8	9	9	11	11	11	11	13	13	13	
Age of patient	6	4	11	3	11	14	4	3	13	3	4	11	15	5	13	14	
Virus present	—	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	
Detroit Throat Washings.																	
Day after onset	All 1 to 3																
Age	7	7*	12	13	15	15	15	16	18		3	4	5	6	7*	7	10
Virus present	(+	—	—	—	+	—	—	+	—)		(+	+	+	—	—	—	+)
	Washing										Swab						

* Washing and Swab combined.

in Dallas, Texas, 1943 and from swabs and washings from patients in Detroit, Michigan during 1944.

Materials and Methods. The washings from patients in Dallas were obtained by expressing 3 to 5 ml of saline into each nostril with a needleless syringe and collecting the fluid in a paper cup after it ran into the throat. The patient then gargled 20 to 30 ml of saline which also were collected in the cup. Washings in Detroit consisted of 20 ml of gargled, distilled water. Swabs were obtained by use of cotton on applicator sticks. These were rolled over the tonsillar areas and the posterior oropharynx then twirled in 1 to 2 ml of saline contained in a lusteroid vial and finally pressed against the side of the tube to express the saline. All specimens were stored under dry ice refrigeration until prepared for inoculation into rhesus monkeys.

Throat washings from Dallas were concentrated by centrifugation of one or two 7 ml samples per specimen in an air-driven centrifuge at 30,000 r.p.m. for one hour.† Supernatant fluid was removed, leaving 0.5-0.75 ml in the tube, and saved for intraperitoneal injection in a monkey that also received the sterile sediment intracerebrally. The sediment was resuspended in the fluid remaining by use of a glass rod; sediments from the companion tubes were combined when there were 2 samples. Bacteria were inactivated by addition of ether which was evaporated *in vacuo* after 1 to 7 days of contact in the refrigerator. In some specimens bacteria survived the treatment with ether and were inactivated by the addition of phenol to a final concentration of 0.5%. One to 1.5 ml of each specimen was injected intracerebrally into a rhesus monkey.

The throat swab fluids from Dallas and the throat washings from Detroit were treated with ether or ether and phenol and inoculated intracerebrally. The latter, however, were first concentrated to a volume of 1 to 2 ml by evaporation from a cellophane "Visking" casing placed before an electric fan.

Results. All monkeys were observed for a period of one month after injection. Animals that developed paralysis were considered positive for poliomyelitis if histological examination of the nervous system revealed characteristic pathology of the disease. The results of the tests are shown in Table I.

Virus was detected in 2 of 16 Dallas patients, one of the spinal and one of the bulbar type. Both specimens were collected on the eleventh day after onset of symptoms. Eleven other specimens obtained between the 3rd and 11th days were negative. Of the specimens collected in Detroit all within 3 days after onset, 3 of 9 throat washings contained virus as did 4 of 7 throat swabs. One of the 4 positive swabs was from a bulbar paralytic and one from a nonparalytic patient.

Discussion. The results obtained tend to support the impression that swabs are as good, if not a better method of recovering virus from the throat than throat washings even though, as in this study, the latter are concentrated before testing.

The evidence to date shows that poliomyelitis virus is present in the throats of patients and may be readily detected early in the illness. The present study duplicates the report of Howe, Bodian and Wenner[5-b] in demonstrating virus in 43% (7 of 16) of those

† The centrifugation was kindly made by Dr. R. W. G. Wyckoff of this laboratory.

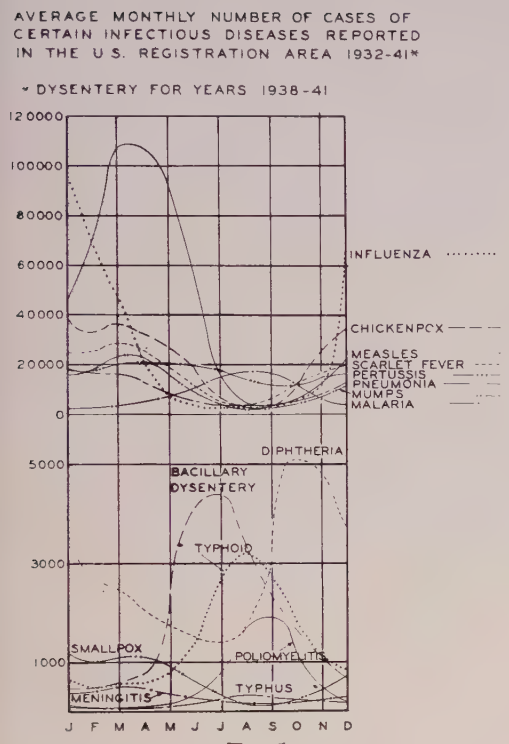

FIG. 1

patients tested within 3 days of onset. However, the fact that virus was detected in two throat washings 11 days after onset suggests that its persistence may be quite variable.

The presence of an agent in the throat does not necessarily mean that the nose and mouth are the chief portals of exit for the germ. For example bacilli are reported present in the mouths of approximately 50% of typhoid patients[9] yet the stools and urine are regarded as the chief routes of contamination from the patient. Likewise the more frequent recovery of poliomyelitis virus from the bowel than from the throat has in recent years tended to emphasize the former as the more important portal of exit.

Another argument favoring enteric over respiratory spread is the seasonal prevalence of the disease. As shown in the figure, poliomyelitis has a seasonal occurrence similar to typhoid, dysentery, typhus and malaria and unlike the disease spread by the respiratory route; diphtheria has a unique curve that perhaps may be explained as resulting in part from increased incidence associated with reopening of schools. The temptation exists to reason from analogy and to suggest that outbreaks of poliomyelitis like those of typhoid-dysentery are started by enteric carriers; the subsequent spread of infection may depend on either or both respiratory or enteric contamination of the environment. More data are needed particularly in regard to the relative importance of the case and of the asymptomatic carrier in spreading the illness and in regard to physiologic effects of nutrition and of environmental temperature on susceptibility to the disease. Meanwhile, until evidence to the contrary is obtained it would seem necessary to consider the throats of poliomyelitis patients as important potential sources of infection.

Summary. Virus was recovered from 4 of 7 throat swabs and from 3 of 9 throat washings of poliomyelitis patients collected during the first 3 days after onset of symptoms. In another series of 16 throat washings collected from 3 to 13 days after onset, virus was isolated from 2 patients 11 days after onset of disease.

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16137

Effects of Gonadotrophic Hormones on Lactation.*†

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The inhibiting effect of steroids on lactation is mediated through the ovaries[1,2,3] and is probably due to the hormonal secretions of the large corpora lutea formed under the influence of the steroids.[4] Since the ovaries are normally stimulated by gonadotrophins of pituitary or chorionic origin, we decided to investigate the effect of these hormones in lactating rats.

It has been reported that gonadotrophins from pregnancy urine inhibit lactation in the mouse and rat; the effective daily dose varies from 10 R.U. to 125 R.U.[5,6,7] Later studies showed, however, that more purified chorionic hormone possesses only a slight inhibitory effect, even when injected at the dose level of 100-500 R.U.;[8,9] its effectiveness can be increased by estrogens.[10] The report that pregnancy urine extracts were active in castrate animals[6] could not be confirmed.[9,11]

Pregnant mare serum has a marked inhibitory influence on lactation and results in the death of the young.[9]

Anterior pituitary extract, although active in adrenalectomized rats treated with cortin[12] has no effect in normal rats.[9] Inhibition of lactation has been obtained in combining such extracts with chorionic gonadotrophins.[13]

The present work was undertaken in order to study the effects of these hormones under the same conditions used previously for the steroids and to see whether some correlations could be established between the ovarian changes elicited by gonadotrophins and the degree of inhibition of lactation.

Forty-one albino rats weighing 250-300 g originating from the same colony and fed exclusively on purina fox chow were used. The number of young in the litters was reduced or brought up to 6 within 24-36 hours of parturition, time at which the treatment was initiated. The mothers were divided into 6 groups treated as follows: Group I, no treatment; Group II, lyophilized beef anterior pituitary preparation (L.A.P.) at the daily dose of 40 mg; Group III, chorionic gonadotrophin (A.P.L.) at the daily dose of 150 I.U.; Group IV, A.P.L. at the daily dose of 150 I.U. during the first 6 days, 300 I.U. from the 6th to the 13th day, and 450 I.U. from the 13th to the 16th day; Group V, pregnant mare serum (P.M.S.) at the dose of 300 I.U.; Group VI, P.M.S. at the dose of 600 I.U. All these preparations in a dry form were suspended or dissolved in a

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† Part of this work was done at the Institute of Experimental Medicine and Surgery.

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TABLE I.
Average Figures on the Growth Rate of Litters and Percentage of Survivals Following Treatment of Mothers.

Groups	Treatment	No. of litters	Initial body wt	Avg body wt on day			% of litters surviving on day		
				5th	10th	16th	5th	10th	16th
1	0	12	6.8	10.6	17.3	26.2	96	96	92
2	L.A.P. 40 mg/day	7	7	12.5	17.5	24.3	97	97	97
3	A.P.L. 150 I.U./day	6	7	10.9	15.8	19.7	100	100	100
4	A.P.L. 150-450 I.U./day	6	7.7	11.7	18.4	21.8	100	100	100
5	P.M.S. 300 I.U./day	5	6.3	11.2	14.8	15.	92	92	18
6	P.M.S. 600 I.U./day	5	6.2	9.6	14.5	13.2	100	71	37

physiologic saline solution; the daily dose was administered in 2 subcutaneous injections. The criteria of efficiency of lactation were provided by the growth curve of sucklings and also by the number of deaths among the young. The mothers were sacrificed on the 17th day, or before, whenever all the young from the same litter died. At autopsy, organs were removed, placed in fixative fluid, then weighed and examined histologically.

L.A.P. had no influence on the growth or the mortality of the young, in spite of a certain degree of toxicity as evidenced by the local reactions near the points of injection. A.P.L. exhibited some inhibitory effect; although the mortality was nil in Groups III and IV, the growth was somewhat retarded, as can be seen from Table I. This effect on growth was more evident from the daily average weight of litters; it reached a maximum around the 14th day, then plateaued or even decreased. P.M.S., the most active preparation, influenced the growth and the mortality of young; the degree of inhibition, however, did not differ significantly whether a dose of 300 or 600 I.U. was administered.

On microscopic examination, only the mammary glands of Group II did not differ from those of Group I (controls). The lumina of ducts and acini were dilated and filled with homogeneous secretion; there was an almost complete disappearance of inter- and intralobular connective tissue.

Mammary glands of Groups V and VI presented a marked variation from the normal controls. There was a marked regression of the glandular tissue. The acini were fairly indistinct and their lumina were filled with a vacuolar material. The acinar cells, which were cuboidal in type with large nuclei, showed no activity. The lobular masses were separated from each other by connective tissue. These glands were not secreting and presented signs of involution.

The picture of mammary glands of Groups III and IV was intermediary between that of controls and that of P.M.S.-treated rats. The acini were not distended by milk and the cells were low cuboidal in type but still secreting. The lobular masses were more distinct due to a decreased activity of the glandular tissue.

As expected, ovaries of all experimental groups were markedly stimulated. The ovarian weights for Groups II to VI were 138, 207, 257, 439 and 263 mg respectively to compare with 56 mg for the controls. On histologic examination the ovaries of Group II were found to contain numerous but small corpora lutea which gave to the organ a mulberry appearance. Those of Groups III to VI were more intensely luteinized; with P.M.S. the corpora lutea were larger than with A.P.L. and in many instances large vesicular or hemorrhagic follicles were observed. Since corpora lutea of the pregnancy type which are associated with inhibition of lactation can be identified by the size of the individual cells, we decided to draw a representative cell of the corpora lutea from each group. From Fig. 1 it can be seen that only the luteal cells of rats treated with P.M.S. presented an hypertrophy similar to that of normal pregnancy.

Although the experimental conditions of our studies and the criteria of activity were somewhat different from those used by others, the results are identical: P.M.S is the most active inhibitor of lactation, then comes

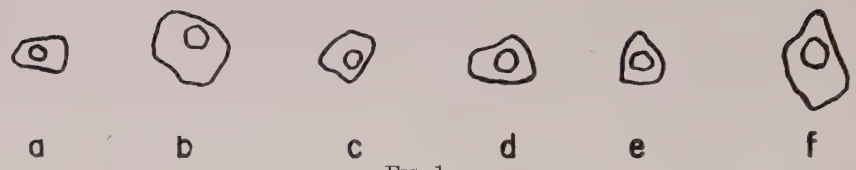

FIG. 1.

Camera lucida drawings of corpora lutea cells. (a) During lactation; (b) during pregnancy; (c) during lactation and A.P.L.; (d) during lactation and L.A.P.; (e) during lactation and Prolactin; (f) during lactation and P.M.S.

A.P.L., while the gonadotrophins of pituitary origin are without effect. It is interesting to note that all the hormonal preparations, especially P.M.S. and A.P.L., produced an intense luteinization of the ovaries; however, only P.M.S. was able to cause the formation of pregnancy corpora lutea. It can therefore be assumed that these large corpora lutea release hormonal substances either different from those secreted by the corpora lutea of lactation or in a different ratio. We tried to see whether the luteotrophic hormone, prolactin, would exaggerate the stimulation of the normally existing corpora lutea. We injected on the day following parturition for a period of 10 days a daily dose of 100 I.U. of prolactin. The growth of the young was normal if not better; the ovaries were slightly heavier (67 mg) than normal and the corpora lutea were well developed but not hypertrophied, as can be seen from Fig. 1. This is in accordance with another observation.[14]

If the small morphologic differences between the A.P.L.- and P.M.S.-treated rats are really associated with different hormonal secretions, the examination of the receptor organs should provide more information regarding the nature and even the level of the hormones in circulation. The vaginae in the 4 groups were mucified, indicating the presence of progesterone. The uteri presented, however, some interesting differences. In the case of the A.P.L.-treated rats the epithelium of the cuboidal type had an hillocky appearance. The stroma was dense, poorly developed and slightly edematous; the cells were darkly stained and fusiform. Glands were present. The general aspect was not much different from that seen in untreated controls although in the latter case this organ was not enlarged. In the P.M.S.-treated rats, the uterine epithelium was tall, not degenerating and presented also many folds. The stroma was well developed and edematous with glands; the cells were large and vesicular with a tendency to being transformed into fusiform cells.

When these observations are compared with those of Atkinson and Hooker,[15] who used the modifications of the endometrium to estimate the level of estrogens and progesterone, it is suggested that in the A.P.L.-treated rats there is a low level of estrogens and progesterone, while on the other hand in the P.M.S.-treated rats there is a high level of estrogens and progesterone. If that be the case, it should be possible to inhibit lactation in ovariectomized lactating rats by giving estrogens and progesterone in a certain ratio; experiments are already in progress to prove that point.

Summary. The influence on lactation of various gonadotrophins of pituitary and chorionic origin has been studied in lactating rats. P.M.S. had a strong inhibitory effect resulting in the death of most of the young; A.P.L. had a slight effect on the growth curve of the young, while an anterior pituitary preparation was inactive. From histologic studies of ovary, vagina and uterus it is suggested that the activity of P.M.S. is due to a high level of estrogens and progesterone.

Our thanks are due to Dr. S. Cook of Ayerst, McKenna and Harrison, Ltd. (Montreal) for A.P.L. and P.M.S., to Dr. H. Jensen of Hormones Corporation (Montreal) for L.A.P., and to Dr. R. McCullagh of the Schering Corporation (Bloomfield, N.J.) for lactogenic hormone.

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16138

Antagonism of Sulfonamide Inhibition by Para-aminobenzoic Acid and Folic Acid in *Toxoplasma* Infected Mice.

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In feeding experiments[1] it has been demonstrated that sodium sulfathiazole and sodium sulfapyridine markedly reduce the fatality rate of mice infected with the protozoan parasite, *Toxoplasma*. Similar inhibitory action has been demonstrated for certain sulfonamide compounds in experimental monkey and avian malaria[2,3] and in experimental avian coccidiosis.[4,5,6]

The mode of action of sulfonamide compounds in the inhibition of multiplication of cells has been studied extensively. According to the Woods-Fildes theory sulfonamide compounds compete with the metabolite para-aminobenzoic acid and thereby interfere with cellular metabolism leading to the inhibition of multiplication of cells. Thus a high concentration of sulfonamide compound may lead to the prevention of cellular multiplication while on the other hand a high concentration of the metabolite can prevent such inhibitory action. This has been demonstrated to be true for various microorganisms both in cultures and in certain *in vivo* experiments. For example, the therapeutic action of sulfanilamide was completely nullified when administered with para-aminobenzoic acid to mice inoculated with *Streptococcus hemolyticus*[7] or *Diplococcus pneumoniae* Type I.[8] In experimental avian malaria,[9,10,11] para-aminobenzoic acid has likewise been shown to have an antagonistic effect toward the therapeutic action of sulfonamide compounds.

Folic acid[12] and certain of its derivatives also have been shown to antagonize the bacteriostatic properties of sulfadiazine in *in vitro* experiments in which *Streptococcus faecalis* Ralston was used as a test organism. In view of this and the fact that folic acid contains a para-aminobenzoic acid moiety it might be assumed that similar results would be obtained in *in vivo* experiments with this compound.

The studies reported here were carried out to determine the effects of para-aminobenzoic acid, folic acid, sodium sulfathiazole and combinations of para-aminobenzoic acid and sodium sulfathiazole and folic acid and sodium sulfathiazole on experimental *Toxoplasma* infection in mice.

Methods. The parasite and maintenance of a stock strain. The R.H. strain of *Toxoplasma* used in all experiments was obtained through the courtesy of Dr. Joel Warren, Army Medical Center, Washington, D. C. in July, 1946. This organism has been maintained in mice by intraperitoneal inoculation of 0.2 ml of a $1x10^{-1}$ dilution of toxoplasma-containing peri-

* This study was supported in part through funds made available by Burroughs Wellcome and Company (USA), Inc.

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TABLE I.

Effect of Different Concentrations of Sodium Sulfathiazole and Para-aminobenzoic Acid on *Toxoplasma* Infection in Mice. Results in the table are based on *the last day in the experiment* on which an accurate LD_{50} titer could be calculated for the control animals.

Compound and conc. in diet	Proportion of inoculated mice dying at dilution of 1x10-1	1x10-2	1x10-3	1x10-4	LD_{50} titer on last day of exp.	Last day of exp.
Sodium sulfathiazole 1.0%	0/7	1/7	1/6	0/4	—0.5	10
Control	7/7	6/7	7/7	3/7	3.93	10
Sodium sulfathiazole 0.15%	0/7	0/7	0/7	0/7	—0.5	9
Control	7/7	7/7	6/7	3/7	3.75	9
Sodium sulfathiazole 0.03%	6/6	2/6	0/6	0/6	1.74	7
Control	6/6	6/6	6/6	0/6	3.50	7
Para-aminobenzoic acid 3.5%	7/7	6/6	5/6	3/7	3.74	10
Control	7/7	6/7	7/7	3/7	3.93	10
Para-aminobenzoic acid 0.15%	5/6	6/6	6/6	3/6	3.83	8
Control	6/6	5/6	5/6	2/6	3.51	8

toneal fluid obtained from previously infected mice. Transfers of the strain have been made every 5 to 6 days.

Titration of the organism and inoculation of experimental animals. Toxoplasma-containing peritoneal fluid obtained from 4 stock mice on the fifth or sixth day of infection was pooled. Serial dilutions of $1x10^{-1}$, $1x10^{-2}$, $1x10^{-3}$ and $1x10^{-4}$ were prepared in Locke's solution. Six to 7 mice, weighing 15 to 20 g each, were inoculated with 0.1 ml of the first dilution and this procedure was repeated for each of the succeeding dilutions using a similar number of mice in each case. The animals were observed for a period of 15 days. Deaths which occurred within 3 days following inoculation are not included in the results. Mice which died between the fourth and fifteenth days were examined for the parasite and included in the data only if the parasite was observed in large numbers in the peritoneal fluid. The LD_{50} titer calculations were made according to the methods of Reed and Muench.[13]

Maintenance of mice during the test period. During the experimental period the mice were fed a standard diet which consisted of sucrose 3650 g, casein (vitamin free) 900 g, Crisco 250 g, salt mixture 150 g,[14] cellu flour Type B 50 g, cod liver oil 75 drops, potassium iodide 75.6 mg, thiamin hydrochloride 25 mg, riboflavin 35 mg, calcium pantothenate 75 mg, niacin 70 mg, pyridoxine hydrochloride 25 mg, and choline chloride 5 g. Test compounds were administered to the animals in this diet. Approximately 4 g of diet or diet-drug mixture was provided for each mouse per day. For each drug tested one group of uninoculated mice was fed the diet-drug mixture thereby serving as a drug control. Water was provided at all times.

Results. Sodium sulfathiazole in a concentration of 1.0% in the diet protected 22 out of 24 mice inoculated with *Toxoplasma* for 15 days. One death occurred in the $1x10^{-2}$ dilution group and one in the $1x10^{-3}$ dilution group. None of the inoculated mice treated with 0.15% sodium sulfathiazole succumbed to the experimental disease. At a concentration of 0.03% protection was slight although deaths occurred somewhat later than in the untreated controls. (Table I)

Toxic effects of sodium sulfathiazole were observed in mice fed a concentration of 1.0% of the drug. These were manifested as lethargy, ruffling of the coat and loss of weight. No toxic effects were noted in animals fed 0.15% or 0.03% sodium sulfathiazole.

Para-aminobenzoic acid at concentrations of 3.5% and 0.15% in the diet had no effect upon the experimental disease in mice. There

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TABLE II.

The Effects of Sodium Sulfathiazole and Para-aminobenzoic Acid, Separately and Combined, on *Toxoplasma* Infection in Mice. Results in the table are based on *the last day in the experiment* on which an accurate LD_{50} titer could be calculated for control animals.

Compound and conc. in diet	Proportion of inoculated mice dying at dilution of 1x10-1	1x10-2	1x10-3	1x10-4	LD_{50} titer on last day of exp.	Last day of exp.
Sodium sulfathiazole 0.15%	0/6	0/6	0/6	0/6	—0.5	8
Para-aminobenzoic acid 0.15%	5/6	6/6	6/6	3/6	3.83	8
Para-aminobenzoic acid + sodium sulfathiazole 0.15% each	6/6	6/6	5/6	0/6	3.39	8
Control	6/6	5/6	5/6	2/6	3.51	8

TABLE III.

The Effect of Sodium Sulfathiazole and Folic Acid Alone and in Combination on *Toxoplasma* Infection in Mice. Results in the table are based on the last day in the experiment on which the accurate LD_{50} titer could be calculated for the control animals.

Drug and conc. in diet	Proportion of inoculated mice dying at dilution of 1x10-1	1x10-2	1x10-3	1x10-4	LD_{50} titer on last day of exp.	Last day of exp.
Exp. I						
Folic acid 0.08%	6/6	6/6	6/6	3/6	4.00	9
Sodium sulfathiazole 0.08%	0/6	0/6	0/6	0/6	—0.5	9
Folic acid + sodium sulfathiazole 0.08% each	6/6	6/6	4/6	0/6	3.24	9
Control	6/6	6/6	6/6	2/6	3.74	9
Exp. II						
Folic acid 0.08%	6/6	5/6	5/5	1/6	3.29	8
Sodium sulfathiazole 0.08%	2/6	2/6	0/6	0/5	1.20	8
Folic acid + sodium sulfathiazole 0.08 % each	4/5	6/6	4/6	2/6	2.29	8
Control	6/6	6/6	6/6	3/6	4.00	8

was no evidence of toxicity resulting from the administration of this compound. (Table I)

When fed a diet containing both sodium sulfathiazole and para-aminobenzoic acid at 0.15% concentration each, the experimental disease in mice was not altered, *i.e.* these animals died at the same rate as untreated inoculated control animals. In this reversal experiment sodium sulfathiazole alone at 0.15% concentration afforded complete protection of all mice at all dilutions of the inoculum while 0.15% para-aminobenzoic acid failed to protect any of the mice. (Table II)

Two experiments were conducted to determine the effects of folic acid and sodium sulfathiazole singly and in combination on *Toxoplasma* infection in mice. A diet containing 0.08% of sodium sulfathiazole prevented the death of inoculated mice while a diet containing 0.08% of folic acid failed to protect any of the mice. When fed a diet containing both drugs at 0.08% concentration each, mice were likewise not protected, *i.e.* animals so treated died at approximately the same rate as inoculated control animals. This experiment was repeated and similar results were obtained. (Table III)

Summary. Mice inoculated with fatal dosages of *Toxoplasma* (R.H. strain) were protected by the administration of sodium sulfathiazole in the diet in concentrations of 1.0%, 0.15%, and 0.08% but not by a concentration of 0.03%.

Neither para-aminobenzoic acid nor folic acid were observed to exert a significant effect on experimental toxoplasmosis in mice.

The protection afforded toxoplasma-infected mice by treatment with sodium sulfathiazole was nullified by the simultaneous administration of para-aminobenzoic acid or folic acid.

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Pharmacological Characteristics of Neohetramine, a New Antihistaminic Drug.* I.

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The symptomatic relief afforded by antihistaminic agents in the treatment of various allergic phenomena has stimulated the search for superior drugs. This search has led to the synthesis of Neohetramine,† 2-(N-dimethylaminoethyl-N-p-methoxybenzyl) amino pyrimidine monohydrochloride. Comparison with other antihistaminic drugs reveals that Neohetramine is relatively non-toxic, and is efficient both in counteracting the effects of histamine and in preventing anaphylactic shock. Preliminary details of this study are set forth below.

Experimental. Acute Toxicity. Acute toxicities of Neohetramine and other antihistaminic drugs were determined in mice (17 to 22 g males, C.F. 1 strain) and guinea pigs (300 to 400 g males). In both species signs of acute toxicity consisted of convulsions, complete extension of fore and hind limbs followed by respiratory paralysis and cardiac arrest. Necropsies performed daily for one week on surviving mice disclosed no gross abnormalities. The LD_{50} doses and associated limits of error, computed graphically,[1] are summarized in Table I. Neohetramine is the least toxic member of the series. In mice, the oral LD_{50} of Neohetramine is approximately twice as high as the intraperitoneal LD_{50}, and in guinea pigs, it is about 5 times as high.

Chronic Toxicity. Neohetramine was administered to 105 weanling rats‡ (approximately 20 per group) either by way of the diet (50, 100, 200 mg/kg daily) or subcutaneously (10, 20 mg/kg twice daily) for a period of 91 days. The experimental animals and 21 (untreated) controls were weighed frequently and complete blood counts were taken at regular intervals. At the end of the test period the rats receiving Neohetramine were indistinguishable from their controls. Treated animals grew at a normal rate, exhibited no abnormalities in blood morphology,

TABLE I.
Acute Toxicity of Neohetramine.

Drug	LD_{50} mg/kg (base)	Limits of error (%)
Benadryl*	74.6	91-110
Pyribenzamine*	65.3	95-106
Hetramine*	61.0	91-110
Neohetramine*	119.0	96-105
" †	245.0	91-110
" † (guinea pig)	493.0	73-137

* Dose given intraperitoneally.
† Dose given orally.

TABLE II.
Protection Against Intravenous Histamine.

Drug	TD_{50}* mg/kg (base)	Limits of error (%)
Hetramine	2.72	85-118
Neohetramine	1.15	64-147
Benadryl	1.00	74-135
Pyribenzamine	0.055	64-148

* Dose protecting 50% of the animals.

TABLE III.
Protection Against Nebulized Histamine.

Drug	TD_{50} mg/kg i.p.	Limits of error (%)
Hetramine	6.4	74-136
Benadryl	3.5	70-143
Neohetramine	3.5	73-140
Pyribenzamine	0.2	65-155
Neohetramine*	3.6	76-132
Pyribenzamine*	0.8	78-128

*Histamine and drug nebulized from same solution.

* We are indebted to Mr. Bernard S. Rubin for his assistance.

† Neohetramine was formerly known as NH-188.

1 de Beer, *J. Pharmacol.*, 1945, **85**, 1.

‡ Rats were maintained on a stock diet of Purina Dog Chow and guinea pigs were maintained on Rockland Rabbit diet.

TABLE IV.
Protection Against Anaphylactic Shock in the Guinea Pig.

Antihistaminic drug*	Dosage (mg/kg i.p.)	No. survivals/ No. animals injected	% survival
None (controls)	—	4/31	12.9
Neohetramine†	5	8/19	42.1
	10	12/20	60.0
	25	10/20	50
Pyribenzamine†	10	13/20	65
	25	14/20	70
Benadryl	25	3/10	30

* Administered intraperitoneally 30 minutes before the shock injection of horse serum.
† The differences between Neohetramine and Pyribenzamine at the 10 and 25 mg dose levels, tested by the Chi Square Method, are not significant.

and developed no organ pathology. We are indebted to Professor George K. Higgins of the New York Medical College for the examination of all microscopic sections.

Protection against intravenously administered histamine. Groups of 10 male guinea pigs (300 to 400 g) were given graded intraperitoneal doses of the various antihistaminics. Thirty minutes later, a uniformly fatal dose (LD_{100}) of histamine diphosphate (0.5 mg per kg histamine) was injected intravenously with the results shown in Table II. In this test Hetramine was the least active, Neohetramine and Benadryl were about equally active and Pyribenzamine was the most active antagonist.

Protection against nebulized histamine. Groups of 10 guinea pigs (300 to 400 g males) were exposed, 4 at a time, to an atmosphere containing histamine aerosol.[2] In these experiments, 0.35 cc of a solution containing 12.5 mg histamine per cc was nebulized in 10 minutes into an 18 liter chamber. Untreated animals invariably died of typical histamine poisoning, but animals exposed 30 minutes after the intraperitoneal injection of increasing doses of the antihistaminic agents were protected as shown in Table III. When tested in this manner Neohetramine has the same potency as Benadryl. Pyribenzamine appears to be 17 times more active than Neohetramine but when, by a slight modification of procedure, histamine and antihistaminic drug were nebulized from the same solution, Pyribenzamine was only 4-5 times as potent as Neohetramine.

Influence on Capillary Permeability. The ability of the various drugs to prevent histamine wheals was determined according to a modification of the method of Last and Loew.[3] Two concentrations of antihistaminic drug (1 and 4 micromols per cc) were combined with equal volumes of histamine in each of 7 concentrations (1, 2, 4, 8, 16, 32, and 64 micromols per cc). Randomized intracutaneous injections of 0.2 cc of each solution were made into the shaved abdominal skin of the rabbit and immediately afterward, 10 cc of 1% aqueous trypan blue was injected intravenously. Histamine antagonism was indicated by failure of the injected site to become blue. The end-point was taken as the highest concentration of histamine antagonized by each dose of antihistaminic drug. An analysis of variance performed on the data of quadruplicate assays indicated that differences attributable to animal variation were nonsignificant. Mean values are, therefore, presented. The data show that the effect of a solution containing 1.9 micromols of histamine per cc (*i.e.*, 0.21 mg) was completely nullified by the incorporation of one micromol of Neohetramine per cc (0.26 mg). In other words, one molecule of Neohetramine antagonized approximately 2 molecules of histamine. Under similar conditions one mole-

2 Loew, E. R., Kaiser, M. E., and Moore, *J. Pharmacol.*, 1945, **83**, 120.

3 Last, M. R., and Loew, E. R., *J. Pharmacol.*, 1946, **89**, 81.

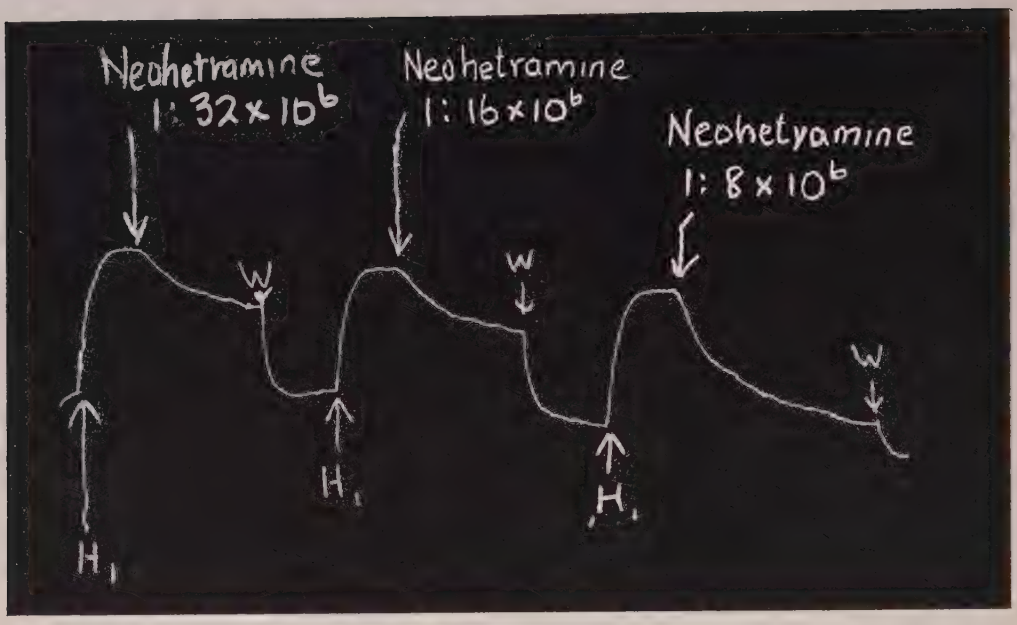

FIG. 1.

cule of Benadryl inhibited 1.5 molecules of histamine and one of Pyribenzamine nullified the action of nearly 9 molecules of histamine.

Excised tracheal tissue. Excised guinea pig tracheal tissue was studied according to the method of Castillo and de Beer.[4] In a 25 cc bath using Hastings-van Dyke solution with glucose, uniform contractions of the tracheal 'chains" produced by 0.72 μg of histamine per cc of bath fluid were reduced by incorporating in the histamine solution graded concentrations of Neohetramine (.029 to 1.876 μg per cc of bath fluid), or Pyribenzamine (.026 to 1.664 μg per cc). As shown in Figure 1, a good linear response was obtained with increasing concentrations of Neohetramine, whereas with Pyribenzamine the response was more erratic. Since the highest drug concentrations were equimolar and reduced the histamine contraction to the same extent (55%), Neohetramine and Pyribenzamine exhibited equal activity against this test object.

Anaphylaxis. The antianaphylactic activity of the various drugs was studied in 140 guinea pigs (300 to 400 g males). Approximately 2 weeks after intraperitoneal sensitization with one cc of 10% horse serum,[5] 27 of 31 untreated animals succumbed to an intravenous shocking dose of 0.5 cc of undiluted horse serum. Intraperitoneal injection of the antihistaminics 30 minutes before the shock dose resulted in survival as shown in Table IV. Protection was obtained with as little as 5 mg of Neohetramine per kg. When 10 mg per kg was injected 60% of the animals survived, but larger doses afforded no greater protection. Pyribenzamine exhibited the same order of activity as Neohetramine, and afforded no greater protection at higher dose levels. Benadryl afforded some protection (30%) at a dosage of 25 mg per kg but 50 mg per kg proved toxic to 3 out of 4 animals. Further experiments involving passive sensitization will be reported in the near future.

Discussion. Wherever possible, the foregoing data were recalculated in terms of the number of molecules of histamine antagonized by one molecule of Neohetramine; the ratios thus obtained varied from 0.5 to 9.0, indicating that the amount of histamine antagonized depended upon the test method employed. Depending on the type of tissue, the species

[4] Castillo, J. C., and de Beer, E. J., *J. Pharmacol.*, 1947, **89**, 104.

[5] Frank, D. E., *J. Immunol.*, 1946, **52**, 59.

of animal, the mode of administration, etc., Pyribenzamine may appear to be one or 20 times as active, and Benadryl may appear to be one-half or equally as active as Neohetramine. The significance of these comparisons is not clear because laboratory methods of testing are as yet unrelated to clinical effectiveness.

In a clinical comparison of 2 antihistaminic drugs, Loveless[6] recently reported that Benadryl was better suited to the treatment of Ménières syndrome and intrinsic allergic asthma while Pyribenzamine was more effective in the treatment of non-seasonal extrinsic asthma. It may be inferred from this report that different drugs will be required in the treatment of different forms of hypersensitivity. Since laboratory methods of testing do not give a reliable index of the clinical value of the antihistaminic drugs, it would appear that final evaluation of these drugs can be established only in human subjects, and then only with reference to the treatment of specific allergic states.

Summary. As judged by the intraperitoneal toxicity in mice, Neohetramine is about one-half as toxic as other antihistaminic agents. Weanling rats, receiving as much as 200 mg of Neohetramine per kg body weight per day over a period of 3 months, grew at a normal rate, exhibited no abnormalities in blood morphology and developed no organ pathology. Neohetramine showed marked activity against the bronchiolar and capillary actions of histamine. Quantitative estimates of the amount of histamine antagonized by Neohetramine varied with the method of testing. Neohetramine conferred protection against anaphylactic shock in guinea pigs actively sensitized to horse serum. The implications of these findings are discussed.

6 Loveless, M. H., *Am. J. Med.*, 1947, **3**, 296.

16140 P

Pharmacological Characteristics of Neohetramine, a New Antihistaminic Drug. II.

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Several compounds which have anti-histaminic properties have been described recently.[1,2] Stress has been laid mainly on their ability to counteract histamine activity and certain allergic phenomena. Many of these compounds possess other properties as shown by their action on smooth muscle and glandular activity.[3,4] Neoantergan[3] is stated to have local anesthetic and quinidine-like properties. Benadryl[4] has some atropine-like activity. Pyribenzamine[5] and 01013[6] enhance epinephrine responses. Described below are some effects of a new anti-histamine compound, Neohetramine.

Its anti-histamine activity was tested on the isolated ileum of guinea pig; isolated uteri of guinea pig, cat, and rat; blood pressure of cats and dogs; the intestine of the cat *in situ*, and blood vessels of the rabbit ear perfused at room temperature. Flow of saliva from submaxillary gland and blood pressure of the cat and dog served to test the effects of Neohetramine on the autonomic nervous system. All excised organs were suspended in

1 Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 703.

2 Winters, C. A., *J. Pharmacol. and Exp. Therap.*, 1946, **87**, 256.

3 Dews, P. B., and Graham, J. D. P., *British J. Pharmacol.*, 1946, **1**, 278.

4 Dreyer, N. B., and Denton, C., *Fed. Proc. Am. Soc. Exp. Biology*, 1947, **6**, 324.

5 Yonkman, F. F., *et al.*, *J. Pharmacol. and Exp. Therap.*, 1946, **87**, 256.

6 Lee, H. M., *et al.*, *ibid.*, 1947, **90**, 83.

oxygenated Ringer fluid kept at 36-37°C. and movements were recorded by the usual lever arrangement writing on smoked kymograph paper. Blood pressure was measured with a mercury manometer.

On the guinea pig ileum and uterus, and uterus of the cat where histamine caused contraction, Neohetramine was capable of abolishing the action of histamine. The amount of histamine causing a submaximal contraction varied from one experiment to another but was kept constant for any particular experiment. Histamine, as base, 0.03 to 0.3 μg per ml gave good contractions of guinea pig ileum. The amount of Neohetramine required to abolish such a contraction was determined and the ratio of Neohetramine to histamine base was calculated. For the cat uterus the ratio was .7:1; for guinea pig uterus, 1.4:1 and for guinea pig ileum, 2.7:1. Neohetramine showed greatest activity on the cat uterus, somewhat less on the guinea pig uterus, and least on the guinea pig ileum.

Neohetramine was less effective in counteracting the action of histamine on the blood pressure of the cat and dog. The fall in blood pressure caused by 1 to 2 μg of histamine was easily offset by 2.5 mg per kg of Neohetramine. However, the vaso-depression caused by larger amounts of histamine, (5-7 μg per kg) was reduced but could not be completely nullified by 8 to 10 mg per kg of Neohetramine. Neohetramine, like Pyribenzamine, was not capable of altering the inhibitory action of histamine on the rat uterus. The inhibition was as marked after Neohetramine as before, even when the ratio of Neohetramine to histamine was as high as 50 to 1. The vaso-constriction of the perfused rabbit ear caused by histamine could be completely counteracted by Neohetramine in a ratio of 1:1.

In atropinized cats and dogs under urethane, or chloralose and urethane anesthesia, Neohetramine (1-5 mg per kg) caused the following changes: an immediate drop in blood pressure without change in heart rate, followed by recovery in a few minutes. This was attributed to cardio-depressant action resulting from a high concentration of Neohetramine in coronary blood. As the drug became generally distributed in the body, the final concentration was insufficient to continue this cardio-depressant action. Large amounts of Neohetramine (8-10 mg per kg) produced a temporary hypotension. Straub heart preparations showed that Neohetramine diminished systolic contraction. As the concentration of Neohetramine in the perfusion fluid was increased, the depression of systole became greater. This was due to direct action on the cardiac muscle and was abolished on washing. Cardiometric measurements on the dog heart showed a diminished systolic and an increased diastolic volume after injection of Neohetramine. On the perfused rabbit ear, Neohetramine caused only slight transient dilatation.

Neohetramine showed little or no effect on sympathetic responses, following injection of epinephrine or sympathetic nerve stimulation. Potentiation of injected epinephrine as described for other anti-histaminics by Yonkman[5] and Lee[6] could not be demonstrated. This lack of potentiation to epinephrine was confirmed on the cat blood pressure and the isolated rabbit uterus. On the parasympathetic nervous system, Neohetramine exerted some atropine-like action on the chorda tympani, but even large doses (8 mg per kg) failed to abolish chorda secretion. Atropine on the other hand (.1 mg per kg) effectively abolished all chorda tympani activity. Vagal inhibition of the heart produced by weak faradization in the anesthetized cat could be eliminated by Neohetramine, but an increase in the strength of faradization tended to restore some vagal inhibition. However, Neohetramine did not lessen the vagal effects on the intestine. In several instances Neohetramine seemed to potentiate vagal contractions of the intestine.

Total and free acidities of gastric juice obtained by rhythmic stimulation of the left vagus were unaltered by Neohetramine, 1-5 mg per kg.

Summary. The above observations indicate that Neohetramine possesses well-marked anti-histaminic properties. In addition it has slight atropine-like actions; it does not alter epinephrine or sympathetic nerve responses.

16141 P

Relation between Time of Fertilization and Follicle Cell Dispersal in Rat Ova.*

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Following the discovery that the enzyme hyaluronidase from sperm disperses the follicle cells of recently ovulated mammalian ova, a concept has arisen that the action of this enzyme facilitates fertilization by denuding the ova prior to or simultaneously with sperm entry.[1-4] The complete cell dispersal seen when mammalian ova are denuded by hyaluronidase *in vitro*[1,3] and the observation of Gilchrist and Pincus[5] who state "the freeing of adherent follicle cells is an inevitable accompaniment of sperm penetration..." have undoubtedly promulgated this concept. Observations to be reported in this note indicate no mass removal of the follicle cells prior to fertilization and that sperm penetration precedes the gross denudation of the ovum in the rat.

Methods. Female rats were bred and 12-26 hours later the ova were removed from the oviducts. The ova were examined in a depression slide for the disposition of the follicle cells and then transferred to a microscope slide to determine if fertilization had occurred. Hyaluronidase (0.1% solution) was drawn under the coverglass to remove the adhering follicle cells when it was necessary to observe the ovum proper.

In another experiment, 0.2 cc of hyaluronidase from bull or rat testes, in concentrations of 30-60 turbidity reducing units per cc, was introduced into each horn of the uterus of a rat in heat and the horns ligated near the cervix to prevent leakage. The enzyme was dissolved in either Ringer's solution or in the uterine fluid which was removed before administering the enzyme.

Experimental. In the first experiment, the ova of 9 rats were observed to be covered with follicle cells and remained in a compact mass when removed from the oviduct. They were similar in appearance to ova recovered from non-bred rats (100 cases). The ova proper could not be dissected free of their follicle cells with dissecting needles. After adding hyaluronidase, which removed the adhering follicle cells from the 65 ova recovered, spermatozoa were identified within the perivitelline space and polar bodies were present in every instance. These fertilized ova were obtained 12-16 hours post-coitus.

From 10 other rats, 31 fertilized ova were recovered, and they were either partially or completely denuded of their follicle cells. A jelly-like matrix surrounded the ova in several instances, but the follicle cells were few and scattered. Dead and motile sperm also were observed in this jelly. The denuded fertilized ova were recovered in two instances 13 hours post-coitus; the remainder at 16-26 hours.

Following the introduction of hyaluronidase into both uterine horns of 12 rats in heat, masses of ova were recovered in every instance 18-24 hours later. In not one case were the ova denuded. Addition of the uterine fluid of these rats to their own ova, *in vitro*, induced complete denudation in 10-20 minutes in all trials.

The results indicate that sperm make their way between the follicle cells and fertilize the ovum before any visible dispersal of the follicle cells occurs. Somewhat later the follicle cells are dispersed, freeing the ova. Furthermore, our observations indicate that

* Aided by a grant from the Sage Fund provided by the Cornell University Trustee-Faculty Committee on Research.

† Fellow of the Schering Corporation.

1 McClean, D., and Rowlands, I. W., *Nature*, 1942, **150**, 627.

2 Joël, C. A., and Eichenberger, E., *Schweiz. Med. Wchnschr.*, 1945, Nr. **27**, 601.

3 Leonard, S. L., and Kurzrok, R., *Endocrinology*, 1945, **37**, 171.

4 Swyer, G. I. M., *Biochem. J.*, 1947, **41**, 409.

5 Gilchrist, F., and Pincus, G., *Anat. Rec.*, 1932, **54**, 275.

the bulk of the sperm and hyaluronidase remain in the uterus following copulation and no mass movement of the enzyme from the uterus into the tubes seems to play a part in the process of denudation and fertilization. We are of the opinion, however, that hyaluronidase is produced by those sperm which gain access to the tubes, and with the aid of this enzyme, the sperm traverse the follicle cell mass to fertilize the ovum. Later the concentration of enzyme is sufficient to denude the ova as was observed 24 hours post-coitus.

Conclusion. Fertilization of the rat ovum occurs before mass displacement of the surrounding follicle cells; denudation of the ova occurs subsequently. Since hyaluronidase, introduced into the uterus, does not pass into the tubes to denude the ova, it seems that only the enzyme associated with the sperm which reach the oviduct disperses the follicle cells.

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Influence of *M. varians* on Oral Infectivity of Mouse-Hamster (M-H) Virus.*

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During the course of experiments on the susceptibility of white Swiss mice (Webster strain) to orally-administered M-H (Mouse-Hamster) virus, an unexplained reduction in the usual mortality of the mice occurred. During the period of January 5 to June 15, 1946 eight experiments involving 214 mice were conducted. The mortality to an orally-administered, highly concentrated mouse-brain suspension of the M-H virus in these 8 experiments varied between 48 and 80%, or an average mortality of 60%. Quite unexpectedly on July 26, the administration of the same dose of the M-H virus that had been used in the previous experiments now produced a mortality of only 20%. No explanation for this reduced mortality was available at that time as the virus end-point by intracerebral or foot-pad inoculation had not altered as compared with the previous 8 experiments. This reduced mortality to the M-H virus occurred again on December 21, 1946 (Experiments 12, 13, Table I). Thus, in the 13 experiments conducted during this year, there were only 2 in which the mortality to the usual dose of the virus was less than 44%-80%, and these mortalities were only one-half to one-fourth of the lowest mortality previously achieved in the other 11 experiments. A review of the protocols of the 13 experiments and the method of preparation of the virus used in the oral feeding experiments shed some light on the problem.

Up to May 15th, consecutive passages of the M-H virus by intracerebral and foot-pad inoculation into the mouse had been performed until a seventh-passage virus was obtained on March 20, 1946. Contemplating a large number of experiments, subsequent to April, 1946, 200 mice were obtained and were inoculated subcutaneously into the foot-pad with a fatal dose of the seventh passage M-H virus. The brains were harvested when paralysis or death occurred, and a 25% emulsion in Ringer's solution was prepared. This total volume of the brain emulsion was then divided into 10 equal portions and stored in sterile lusterloid tubes in the CO_2 icebox at -40° C until needed in the future. Thus, the first experiment conducted with one of the samples of this new batch of virus was done in May, 1946, approximately 4 days after

* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I.

Mortalities of 13 Groups of Swiss Mice Fed M-H Virus Brain Suspension. The concentration of the virus suspension fed was 10% except in Exp. 4 and 5 when 2.5% was used. The virus end-point, by subcutaneous injection, in Exp. 1 to 11 was 100% of the animals when 0.03 cc of 10^{-6} dilution was given. In Exp. 12 and 13 the end-point was 10^{-7}.

Exp. No.	Date	Virus			Mice			Mortality, %
		Amt fed	Passage	No. of feedings	Age, days	Sex	No.	
1	1– 5–46	.5–.9	3	1	28	Mixed	26	65
2	25	.5–.9	4	1	180+	Males	50	48
3	2–13	.5–.9	5	1	50	Mixed	40	47
4	18	.5–.9	6	3	28	”	25	80
5	18	.5–.9	6	3	28	”	25	48
6	3–24	.5–.9	7	2	52	”	10	60
7	5–19	.5	8	1	28	”	18	77
8	6–15	.5	8	2	40	”	20	55
9	7–26	.5	8	3	48	”	20	*20*
10	9–21	.5	8	1	48	Males	20	45
11	21	.5	8	1	48	Female	27	44
12	12–21	.5	8	2	48	Males	28	*17.8*
13	21	.5	8	2	48	Female	20	*10*

the preparation of this batch of virus. The end-point of this fresh virus by foot-pad inoculation was still 10^{-6} and the mortality to the same large oral dose was 77% (Experiment 7, Table I). One month later on June 15, 1946, a second vial of the virus suspension was removed from the CO_2 icebox for the conduction of Experiment 8 (Table I). At this time the usual high mortality of 55% was obtained with the same dose and concentration of the M-H virus used previously. However, on July 26, 1946, a third tube of the virus suspension prepared in April was removed from the icebox for the conduction of Experiment 9 (Table 1). In spite of the fact that in this experiment the mice received 3 consecutive feedings of the same dose of virus as in previous experiments the mortality was only 20%. On Sept. 21, 1946, another vial was removed from the icebox for Experiments 10 and 11, and a good mortality was obtained. Again on Dec. 21, 1946, when Experiments 12 and 13 were conducted, mortalities of only 17.8% and 10% were obtained.

Fortunately, there was remaining from Experiments 12 and 13 (Table I) a small amount of the original 25% mouse-brain virus suspension that had been used in these 2 experiments. It was noted that this suspension had a rather orange or amber color and that particulate matter had settled out. For this reason the material was cultured on various media, and an organism with the following properties was found. This organism tolerated a temperature of 60° C for 30 minutes. It reduced nitrates to nitrites and fermented all of the usual 6-carbon sugars, dextrins and glycerol with the production of acid only. Starch was not hydrolyzed. The inoculation of pure cultures of the organism intraperitoneally and intracerebrally into white mice produced no signs of peritonitis or meningitis. The mice remained well for the 14-day observation period subsequent to inoculation. The organism grew well on nutrient agar, the colonies having a faint yellow color when grown at room temperature, but not when grown at 37° C. When grown in a mouse-brain suspension in Ringer's solution at 4° C or 11° C the colonies at the meniscus have a faint pinkish tinge. The brain suspension also developed a slight orange-pinkish color. This organism has been identified as *Micrococcus varians*.

A relationship of this organism to the reduced oral infectivity of the M-H virus was postulated, and the following experiments were done.

An amount of eighth-passage virus, (that used in Experiments 12 and 13 and containing the *Micrococcus varians* sufficient to feed 18 mice was left. This was prepared as a 10% suspension and fed in 0.5 cc amounts for 2

TABLE II.

Comparison of the Oral Infectivity and the Subcutaneous Foot-pad Titer of Eighth and Ninth Passage M-H Virus. A 10% brain virus suspension was fed. Eighth passage virus contained *Micrococcus varians*, ninth passage virus was sterile by culture.

Mice			Virus				
Sex	Age (days)	No.	Passage No.	Amt fed	No. feedings	Subcutaneous end point	Mortality, %
Mixed	32	18	8	0.5	2	10^{-6}	16.6
"	32	18	9	0.5	2	10^{-4}	33.0

consecutive days. For controls, a small amount of this virus was passed subcutaneously into young Swiss mice whose brains were harvested in order to obtain a ninth-passage virus, free of the organism. Thus, a comparison of the original micrococcus containing eighth-passage virus, and micrococcus free ninth-passage virus could be made. The eighth-passage virus material produced a heavy growth of the micrococcus by aerobic culture while the ninth-passage virus material was sterile. A titration, by foot-pad, of each of the viruses was done, and a suitable group of animals, (18 in number), was fed an 0.5 cc dose of this ninth-passage virus as a 10% suspension.

Table II presents the results obtained in this experiment. The 18 male mice, fed on 2 consecutive days, a dose of 0.5 cc of the 10% eighth-passage virus, had a mortality end-point by foot-pad inoculation of 10^{-6} and a mortality of only 16.6% to oral administration of the virus. On the other hand, the mice fed the same dose of ninth-passage virus, which had the higher oral infectivity, had a lower end point (10^{-4}) by foot-pad inoculation.

Table III presents the results of a more critical experiment in which the mortalities of Swiss mice fed the following preparations were determined: (1) 10% normal mouse brain suspension. (2) 10% normal mouse brain plus 1 cc of *Micrococcus varians* culture. (3) 10% M-H virus mouse brain suspension. (4) 10% M-H virus mouse brain suspension plus 1 cc of *Micrococcus varians* culture.

The method of preparation of the mouse brain suspensions for this experiment was as follows: 10% normal mouse brain in Ringer's solution was prepared. One hundred cubic centimeters of this solution was divided into 2 aliquots, each aliquot containing 50 cc. To one aliquot, 1 cc of a 25 day culture of the micrococcus was added. (This preparation of the *Micrococcus varians* was prepared from 20% guinea pig brain suspension in which the micrococcus had been inoculated and incubated for 25 days at 11° C). The second aliquot of the normal mouse-brain suspension received no *Micrococcus varians* inoculum. These 2 brain suspensions served as the controls. The brain suspensions containing the M-H virus were prepared by making 100 cc of a 10% M-H infected mouse-brain suspension. This volume was divided into 2 aliquots of 50 cc each. The first aliquot was inoculated with 1 cc of the *Micrococcus varians* culture while the second aliquot of the M-H brain suspension was not inoculated. All 4 aliquots were then cultivated, both aerobically and anaerobically, on blood agar plates at the time of preparation and all were found to be sterile except the 2 which had been inoculated with the micrococcus. These 4 specimens of mouse brain; namely, (1) normal mouse brain, (2) normal mouse brain plus *Micrococcus varians*, (3) M-H brain, and (4) M-H mouse brain plus *Micrococcus varians* were incubated at 11° C for 29 days. Ten days after the inoculation of the brain suspension with the *Micrococcus varians*, all of the tubes were cultured on blood agar. Growth existed only in the *Micrococcus varians* inoculated tubes. The other 2 control tubes were sterile. Twenty-nine days after inoculation with the micrococcus, the 4 tubes of mouse brain were fed to 4 groups of normal male mice. Each mouse was given 0.5 cc of the 10% mouse-brain suspension for three

TABLE III.
Influence of *Micrococcus varians* on the Oral Infectivity of the M-H Virus.

	No. of mice	Mortality
Normal brain suspension	25	0
" " " + *Micrococcus varians*	25	0
M-H brain suspension	26	65.0
" " " + *Micrococcus varians*	24	33.3

TABLE IV.
Comparison of the Inhibiting Effect of *M. varians* Organisms and *M. varians* Culture Filtrate on the Oral Infectivity of the M-H Virus.

	No. of mice	Mortality, %
M-H virus + *M. varians* (incubated 97 days)	21	23.8
M-H virus + washed *M. varians* (incubated 48 hr)	20	35.0
M-M virus + *M. varians* filtrate (incubated 48 hr)	20	50.0
M-H virus only	17	59.4

successive days. These experiments presented in Table III demonstrated that when the M-H virus was incubated with a growing culture of the *Micrococcus varians* its oral infectivity was reduced by one-half.

A virus titration of the specimen without bacteria was carried out by foot-pad inoculation at the time the brain samples were prepared and when fed. Likewise, the normal mouse brain and the normal mouse brain containing the *Micrococcus varians* were inoculated into 5 mice at the time of feeding. All mice survived.

At the time of the feeding of the virus, samples of brain suspension were removed from the bottom of each of the 4 tubes and cultured on individual blood agar plates. *Micrococcus varians* was found only in the 2 samples that had been inoculated 29 days previously.

After incubating for 29 days the sample of brain suspension containing the virus only and the sample of virus brain suspension which had been inoculated with the *Micrococcus varians* were titrated by foot-pad method in replicates of 5 each of 4-week-old mice. The volume of the dilution inoculated into each mouse was 0.03 cc. All samples of the M-H virus brain suspension had the same end point; a subcutaneous dose of 0.03 cc diluted to 10^{-7} killed 100% of the animals.

The following experiment was done in an attempt to determine whether or not this reduction in mortality was due to a direct action of the micrococcus on the virus and thus to a reduction in the number of virus particles available for absorption, or perhaps due to the action on the virus of some agent in the culture filtrate produced by the micrococcus. Four groups of 8-week-old male mice were fed for 2 consecutive days 0.5 cc of the following 10% mouse brain virus suspension: (1) M-H brain-virus suspension plus micrococcus organisms (incubated at 4° C for 97 days). (2) M-H brain-virus suspension plus washed *Micrococcus varians* organisms (incubated at 4°C for 48 hours. (3) M-H brain-virus suspension plus *Micrococcus varians* filtrate (incubated at 4°C for 48 hours). (4) M-H brain-virus suspension only. These results are tabulated in Table IV.

Both the M-H virus suspensions containing the *Micrococcus varians* organisms had a lower mortality than that obtained with the M-H virus brain suspension alone. The filtrate from the *Micrococcus varians* culture did not significantly reduce the infectivity of the M-H virus. It appears that the action of the *Micrococcus varians* organism on the M-H virus is a direct one, possibly related to its metabolic activity or to some absorption phenomenon.

Summary. Suspensions of mouse brain tissue infected with the M-H (Mouse-Hamster) virus when incubated with a pure culture of the *Micrococcus varians* organism were found to have a markedly reduced oral infectivity for the white Swiss mouse. In spite of this reduction in oral infectivity there is no change in the infectivity of the virus material con-

taining the micrococcus when it is injected subcutaneously. Filtrates of the micrococcus culture do not appear to inhibit the oral infectivity of the virus. It is suggested that the M-H virus may have been absorbed on the *Micrococcus varians* organism, and is thus not as readily available for invasion through the intestinal tract or that the virus itself may have been modified so that its oral infectivity has been reduced while its infectivity by subcutaneous inoculation is unchanged.

16143 P

Allantoin Clearance as a Measure of Glomerular Filtration Rate in Man.*

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In a recent study,[1] the renal clearance of allantoin in the rat and dog was found to be equal to that of creatinine. This equivalence held true despite variations in both urine flow and plasma concentration of allantoin. It seemed probable therefore that the clearance of allantoin could serve as a measure of glomerular filtration rate in these two species. Furthermore, it seemed possible that the clearance of this same substance might serve as a measure of glomerular filtration rate in man. Therefore it was thought advisable to determine the renal clearance of allantoin in normal human subjects.

Methods. Five normal men and one woman received 10 g of allantoin dissolved in 500 cc of fresh orange juice by mouth at 8:00 A. M. on the day of the experiment. Each subject was kept at bed rest until 9:30 A. M. at which time they were catheterized and given 1000 cc of H_2O by mouth. At 10:00 A. M., the bladder was emptied, washed out with normal saline solution, a blood sample taken and the first urine collection begun. At 10:30 A. M., the bladder was emptied again, washed out with saline solution, and a second blood sample was obtained. The second urine collection began immediately after the bladder had been emptied and washed. At 11:00 A. M., the second and final collection period was terminated, and a third blood sample was obtained.

Urine and plasma samples were analyzed for allantoin by the method of Christman, Foster and Esterer,[2] modified as described in a previous study.[3]

The allantoin clearance of each subject then was calculated as an average of the clearances obtained for the 2 collection periods and corrected to 1.73 square meters of surface area.

Results. As Table I indicates, the allantoin content of plasma, 120, 150 and 180 minutes after oral ingestion of 10 g of this substance remained approximately constant. The average stability of the plasma allantoin during the entire clearance study of course eliminated any need for supplementary oral or parenteral administration of allantoin.

The average plasma allantoin clearance of the 6 subjects was found to be 123 cc per minute (See Table I). Individual clearances varied from 107 to 137 cc per minute. It should be mentioned that no ill effects were observed in any patient during or after the clearance study.

Discussion. Similar to the results obtained in the rat and dog,[1] the present study indi-

* Aided by a grant from the U. S. Public Health Service.

1 Friedman, M., and Byers, S. O., *Am. J. Physiol.*, to be published.

2 Christman, A. A., Foster, P. W., and Esterer, M. B., *J. Biol. Chem.*, 1944, **155**, 161.

3 Byers, S. O., Friedman, M., and Garfield, M. M., *Am. J. Physiol.*, to be published.

TABLE I.
The Renal Clearance of Allantoin in Human Subjects.

Patient	Age, yr	Avg urine flow (cc/min)	Plasma allantoin conc. (mg/100 cc)			Renal allantoin clearance (cc/min)		
			10:00	10:30	11:00	Per. I	Per. II	Avg
W.J.	45	13.45	7.4	7.0	7.5	101	113	107
H.B.	22	3.70	6.1	7.1	6.8	124	136	130
G.C.*	43	10.00	5.5	5.1	4.3	134	140	137
H.S.	43	5.70	6.9	7.7	8.8	132	114	123
R.S.	42	5.20	5.3	4.4	5.1	115	114	127
D.C.	32	5.90	4.3	4.3	4.8	131	123	115
Avg		7.33	5.9	5.9	6.2	123	123	123

* Female.

cates that the clearance of allantoin probably measures the rate of glomerular filtration in man. Thus, the average allantoin clearance obtained in the 6 subjects (123 cc per minute) is approximately the same as the value found for the inulin clearance (123 cc per minute) in man by Smith, Goldring and Chasis[4] and by one of us (M.F.) in a previous study.[5]

Conclusion. The average renal clearance of allantoin in 6 normal human subjects was found to be 123 cc per minute. The similarity of this value to that now accepted for the renal clearance of inulin (a glomerular filtrate without tubular reabsorption) strongly suggests that the renal clearance of allantoin is at the level of glomerular filtration.

4 Smith, H. W., Goldring, W., and Chasis, H., *J. Clin. Invest.*, 1938, **17**, 263.

5 Friedman, M., Selzer, A., and Rosenblum, H., *J. A. M. A.*, 1941, **117**, 92.

16144

Effect of Folic Acid* and Bis (β-Chloroethyl) Sulfide (Mustard Gas)† on Transplanted Mouse Lymphosarcoma.‡

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Leuchtenberger *et al.* [1,2] have shown that folic acid is a tumor growth inhibitor for sarcoma 180 and for spontaneous mammary tumors in mice. Bass and Freeman[3] were not able to demonstrate a similar regression in transplanted lymphoma $6C_3HED$ in mice. The present investigation was designed to further study the effect of folic acid on the $6C_3HED$ tumors, when administered alone or in combination with bis (β-chloroethyl) sulfide. It was hoped that the folic acid might reduce the severity of the toxic side effects of bis (β-chloroethyl) sulfide. Although this did not prove to be the case, certain interesting observations were made which are reported in this paper.

* The "synthetic folic acid," "Folvite," and "Teropterin" used were supplied by Lederle Laboratories, Pearl River, N.Y.

† The bis (β-chloroethyl) sulfide was supplied by Edgewood Arsenal, Md.

‡ These studies have been made with the assistance of a grant-in-aid from the National Cancer Institute.

1 Leuchtenberger, C., Lewisohn, R., Laszlo, D., and Leuchtenberger, R., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 204.

2 Leuchtenberger, R., Leuchtenberger, C., Laszlo, D., and Lewisohn, R., *Science*, 1945, **101**, 46.

3 Bass, Allan D., and Freeman, Marion L. H., *J. Nat. Cancer Inst.*, 1946, **7**, 171.

Methods. Adult C_3H mice of both sexes obtained from Jackson Memorial Laboratories at Bar Harbor, Maine and Lymphosarcoma $6C_3HED$ were used. Small fragments of tumor were implanted subcutaneously into the right axillary region with a 16-gauge needle. When the tumors were large enough to measure, treatment was started. Ninety animals were used for this study; 30 receiving bis (β-chloroethyl) sulfide alone, 30 synthetic folic acid alone, and 30 both the bis (β-chloroethyl) sulfide and folic acid.§ Folic acid was administered in daily doses of 0.1 mg per mouse. The bis (β-chloroethyl) sulfide was given in doses of 3 mg per kg at 48-hour intervals unless the animals developed toxic symptoms in which case the interval was prolonged or the dose reduced to fit the individual case. Diarrhea and/or a weight loss of 1 g or more in 24 hours were used as criteria for varying the procedure until cessation of such symptoms. Both folic acid and bis (β-chloroethyl) sulfide were administered intraperitoneally; folic acid in a phosphate buffer solution of pH 7.1 and bis (β-chloroethyl) sulfide in 0.076% by volume solution in propylene glycol. All animals were fed Purina dog chow *ad libitum*.

Results. Fig. 1 summarizes the results obtained. Since bis (β-chloroethyl) sulfide is lethal for some animals at the dosage used, the points plotted on the curve represent the average tumor size of surviving animals. Tumor size is indicated as one half the length plus the width expressed in centimeters. Tumors of animals receiving bis (β-chloroethyl) sulfide showed a diminished rate of growth with a definite period of regression on the sixth day after the treatment was begun. Tumors of animals receiving both folic acid and bis (β-chloroethyl) sulfide clearly showed less effect of the therapy than when bis (β-chloroethyl) sulfide alone was administered. Although less regression or growth inhibition was evident in the animals receiving both bis (β-chloroethyl) sulfide and folic acid the survival time was not significantly altered. The rate of growth of the tumors in control animals receiving phosphate buffer or propylene glycol was approximately the same as the tumor growth rate of mice receiving folic acid (Fig. 2).

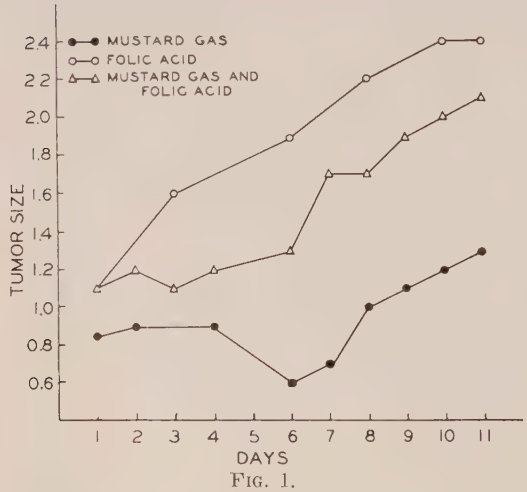

FIG. 1.

The indicated treatment was begun on the first day shown in graph. This is approximately 7 days after transplantation.

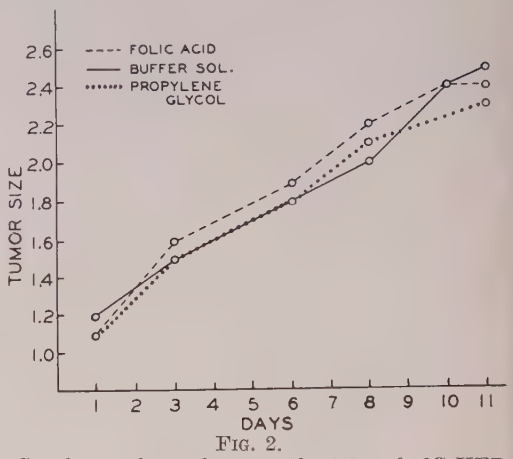

FIG. 2.

Graph to show the growth rate of $6C_3HED$ tumors in control animals as compared with animals receiving "synthetic folic acid." Propylene glycol and phosphate buffer were given intraperitoneally to 10 animals each. Thirty animals received folic acid.

To determine whether there was any difference in growth rate of lymphoma $6C_3HED$ when tumor-bearing mice were given pteroyl mono-glutamic acid or pteroyl tri-glutamic acid, we gave 15 C_3H mice 0.1 mg of "Folvite" daily in phosphate buffer and another 15 mice 0.1 mg of Teropterin (pteroyl tri-

§ Unless otherwise stated, folic acid in this paper will refer to "synthetic folic acid" (Lederle).

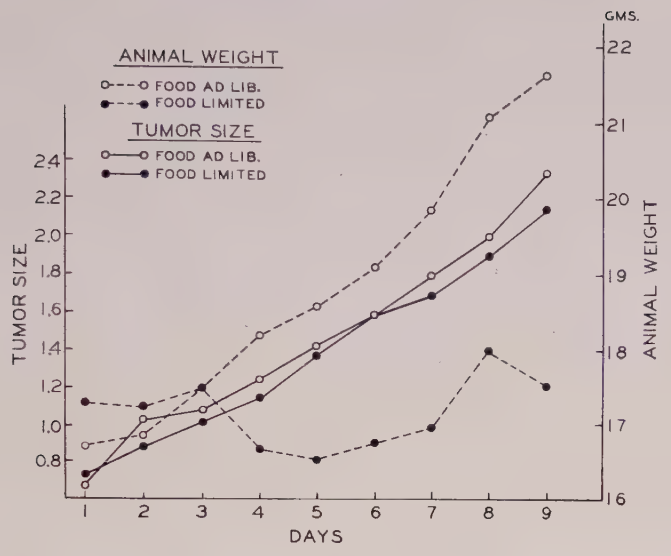

FIG. 3.
Food limitation was begun on the second day.

glutamic acid) daily for 5 days. The growth curve of the tumor in these two groups was not significantly different from controls treated with phosphate buffer alone, or from the growth curves when "synthetic folic acid" was used.

The weights of the animals receiving both bis (β-chloroethyl) sulfide and folic acid were maintained better than those receiving bis (β-chloroethyl) sulfide alone. To exclude the possibility of weight loss associated with drug administration as the cause for the differences in tumor regression a paired feeding experiment was carried out, the results of which are shown in Fig. 3. Here it is shown that limitation of food to 60% of that eaten by the control mice has no measurable effect upon the tumor growth. The weight loss produced by the food limitation is comparable to that which is frequently encountered after bis (β-chloroethyl) sulfide administration. Additional studies are necessary to elucidate the mechanism of folic acid antagonism to bis (β-chloroethyl) sulfide.

Summary. "Synthetic folic acid", "Folvite," or "Teropterin" in the dosage employed do not produce regression in transplanted $6C_3HED$ tumors in C_3H mice. Administration of synthetic folic acid partially inhibits the effect of bis (β-chloroethyl) sulfide on lymphosarcoma $6C_3HED$.

16145 P

Activation of Staphylocoagulase.*

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Smith and Hale[1] have shown that the inability of staphylocoagulase to clot citrated plasmas is, in most cases, due to absence of an "activator substance" for the staphylo-

* This work is the fifth of a series of studies on "Enzymes and Enzyme Inhibitors in Relation to Blood Coagulation and Hemorrhagic Diseases," aided by a grant from the John and Mary R. Markle Foundation.

† Junior Research Fellow, U. S. Public Health Service.

coagulase. Gratia[2,3] originally showed that staphylocoagulase clotting is independent of prothrombin activation, and Rigdon's[4] recent evidence, using heparin, confirms the inability of antithrombic agents to inhibit staphylocoagulase clotting. We have repeatedly confirmed these facts and present the following data as a preliminary report of progress in identifying the "activator" in various plasma, serum, and other materials.

Materials and methods. 1. STAPH.: Staphylocoagulase was prepared by precipitating 48-hr. *Staphylococcus aureus* (stock strains) broth-culture filtrate (Berkefeld *N*), or centrifugate, with 3 volumes of 95% ethyl alcohol, at 0°C. The method is essentially that by which Tillett and Garner[5] prepared "fibrinolysin" (*streptokinase*[6]). The ability to obtain an active staphylocoagulase from simple broth culture filtrates confirms Smith and Hale[1] and opposes Lominski,[7] whose results were negative unless plasma was added to the culture medium. A typical yield from 850 cc broth culture filtrate approximates 1.5 g of grayish-brown powder, completely soluble in 1% solution in our citrated borate buffer. The powder, stored in refrigerator (5°C) for 8 months, showed no loss of activity and its solutions were stable for as long as 10 days at room temperature. Reagents previously described:[6] 2. buff. (cit.): borate buffer (plus 0.4% sod. citrate; pH: 7.55), 3. B.F.: (Armour's) bovine plasma fraction-I (50% fibrinogen), 4. H.F.: (Harvard) human plasma fraction-I (35% fibrinogen),‡ 5. PRO.: (Seegers') purified bovine prothrombin.

Additional reagents: 6. H.Pl.: human plasma, 'Lyovac' dried (Sharp and Dohme), 7. B.f.: bovine fibrinogen (Seegers[8]), 8. Ac.G.: "accelerator globulin" from bovine plasma, courtesy Dr. W. H. Seegers[9] (Wayne Univ.), 9. Factor V: (after Owren[10]) prepared from dog plasma, 10. H.Pl.G. and H.S.G.: human 'globulin,' fractionated by 50% sat. $(NH_4)_2SO_4$ from (a) H.Pl. (*v.* 6) and (b) serum from same after clotting with $CaCl_2$, 11. H.Pl.A. and H.S.A.: human plasma (a) and serum (b) 'albumin,' fractionated from supernatants of above at 100% sat. $(NH_4)_2SO_4$, 12. H.S.A. cryst.: crystalline human serum albumin (Harvard Labs.),‡ 13. tpln.: commercial thromboplastins—I. Difco, II. Maltine, prepared according to directions.

Results. Table I describes staphylocoagulase tests with human plasma and the globulin and albumin fractions from plasma and serum. Bovine fraction-I (fibrinogen), plasma, and buffer (without STAPH.) do not clot (2) and B.F. has no activator substance (3), which must be supplied by the addition of plasma (1). The globulins from plasma (5) or serum (9) contain a weak thrombin, but the definite clotting improvement in the presence of staphylocoagulase (4,8) indicates some activator substance also. Crude albumins from plasma (6) or serum (10) are the best single source of activator substance, and are not coagulant in the absence of staphylocoagulase (7,11). Further investigation of these albumin fractions is currently being undertaken to yield more information on the staphylocoagulase mechanism.

Table II lists other substances tested by the same means as in Table I, except for

1 Smith, W., and Hale, J. H., *Brit. J. Exp. Path.*, 1944, **25**, 101.

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4 Rigdon, R. H., and Haynes, A., *Ann. Surg.*, 1942, **116**, 430.

5 Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485; 1934, **60**, 239.

6 Ferguson, J. H., Travis, B. L., and Gerheim, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 285, 302.

7 Lominski, I., *Nature*, 1944, **154**, 640.

‡ The human plasma fractions used in this work were prepared from blood collected by the American Red Cross under a contract between the Office of Scientific Research and Development and Harvard University, and supplied through the courtesy of Drs. Cohn, Edsall, Minot, and colleagues.

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9 Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

10 Owren, P. A., *The Coagulation of Blood: Investigations on a New Clotting Factor* (Oslo), 1947, p. 327.

TABLE I.
Staphylocoagulase Tests with Plasma and Serum Fractions.

No.	B.F. (1%) (0.5 cc)	Buff. (cit.) (0.25 cc)	Staph.† (0.25 cc)	Plasma fraction (0.5 cc)	Clotting-times (min., at 39°C)* 1+	2+	3+	4+
1	+	—	+	H.Pl.	21′	78′	—	107′
2	+	+	—	H.Pl.	No clotting			
3	+	+	+	—	,,	,,		
4	+	—	+	H.Pl.G.	13′	—	78′	163′
5	+	+	—	H.Pl.G.	40′	(clot never complete)		
6	+	—	+	H.Pl.A.	21′	—	33′	46′
7	+	+	—	H.Pl.A.	No clotting			
8	+	—	+	H.S.G.	—	—	21′	42′
9	+	+	—	H.S.G.	—	—	21′	92′
10	+	—	+	H.S.A.	—	—	—	17′
11	+	+	—	H.S.A.	No clotting			

† Staph.: Lot No. 5-6.
* Degree of clotting expressed as follows:
1+: first appearance of weak clot;
2+: weak clot with considerable liquid;
3+: good clot with a little liquid but too soft to invert tube;
4+: solid clot, tube invertible.

TABLE II.
Tests for "Activator Substance" of Staphylocoagulase.

No.	Material tested (0.5 cc)	B.F. (1%) (0.5 cc)	Staph.† (0.25 cc)	Clotting-times (39°C) 1+	4+
1	H.S.A., cryst. (1%)	+	+	No clotting (48 hr)	
2	PRO. (1%)	+	+	,, ,,	,,
3	Factor V (1%)	+	+	,, ,,	,,
4	Ac.G. (1%)	+	+	,, ,,	,,
5	H.F. (1%)*	—	+	20′	40′
6	B.f. (1%)*	—	+	40′	overnight
7	tpln——I (5%)	+	+	—	240′
8	tpln.—II (2.5%)	+	+	—	-60′

† Staph.: Lot No. 5-4.
* Double volumes (1.0 cc) of H.F. or B.f. used, and B.F. omitted.

substituting the respective material for the plasma fraction. The negative results with (1) *crystalline* human serum albumin, (2) purified prothrombin, and the recently identified globulin accelerators of prothrombin conversion (thrombic clotting), *viz.* (3) Owren's factor V and (4) Seegers' Ac.G., are noteworthy. The impure fibrinogens (5,6), which contain some activator substance, should be compared, in this respect, with B.F. (Table I, 3). With enough citrate in our test system to prevent possible thrombin formation (only negligible traces of prothrombin occur in B.F.,[6] however), the evidence for accelerator substance in the thromboplastins (7,8) is interesting.

Summary. The preparation and some properties of a stable staphylocoagulase are described, together with data to indicate that the "activator substance" which it needs in order to coagulate fibrinogen can be provided by certain plasma protein fractions, particularly being associated with the albumins.

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Streptomycin in Experimental Plague.*

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In July, 1944, Dr. Selman A. Waksman invited a study of the therapeutic properties of streptomycin in *P. pestis* infections. Preliminary tests with a one g sample of the crude antibiotic gave very promising results. Subsequently, larger lots of streptomycin were obtained through the generosity of Dr. I. M. Carlisle, Merck and Co., Inc., Rahway, N. J., and in 1945 a confidential report of results was made to the Committee on Medical Research of the Office of Scientific Research and Development. Publication times for that report and later studies are indefinite, but the more significant results are summarized here.

(1) *Results obtained in vitro.* 1,250 μg/cc streptomycin in hormone broth kills 100 million virulent *P. pestis* (Shasta) in 15 minutes. The same number of organisms are destroyed in 4, 12, 24, 48 and 120 hours, respectively, by 313 μg/cc, 78 μg/cc, 39 μg/cc, 20 μg/cc and 5 μg/cc. One hundred thousand organisms of a recently isolated human strain (Modoc) were killed by 0.2 μg/cc, and an equal amount of the "Shasta" strain by 1.9 μg/cc in 72 hours. Amounts varying from 0.4 to 4.0 μg/cc proved bactericidal to 6 plague strains of Hawaiian, Egyptian, Indian and Californian origin in 5 days. From 1 to 16 units/cc of dihydrostreptomycin are required to sterilize these strains in 5 days. Avirulent strains are more resistant: for example, the E.V.76 plague bacillus (Girard)[1] is killed in the presence of 16 units/cc/5 days, strains 14 and 1122 (Jawetz and Meyer)[2] by 8 units/cc and the Tjiwidej strain (Otten)[3] by 4 units/cc, while in the presence of 10% blood serum, 40 units/cc were required to kill 10,000 virulent *P. pestis* "Yreka" in 48 hours. Other factors, such as the chemical composition and the pH of the suspending medium, the concentration of the antibiotic, and the age and density of the culture, influence the bactericidal activity of streptomycin on *P. pestis*. 5,000 μg/cc in broth destroyed 22,000 million virulent *P. pestis* (Yreka)/cc, while the same concentration sterilized 33,000 million/cc in physiologic saline. Four virulent strains trained to resist streptomycin in the amount of 5,000 μg/cc were avirulent and resisted only 2,500 units/cc dihydrostreptomycin.

(2) *Effect on mice and guinea pigs infected subcutaneously.* The relative sensitivity of *P. pestis* to streptomycin permits effective therapy of experimental bubonic disease caused by 100 to 1,000 multiples of the M.L.D. in highly susceptible (ABC) mice. Treatment usually begins on the 48th hour after subcutaneous introduction of the bacilli, when infection is generalized, a bacteremia is well established in 40 to 60% of the animals, and the immunity mechanism is partially damaged by toxins. Irrespective of dosage or frequency of administration, sulfonamides save an average of only 35% of mice at this stage of infection. In 70 separate experiments, treating as many groups of 20-50 mice with varying amounts of streptomycin, advanced experimental bubonic plague was completely cured with 500 μg/3 hours for 3 days, or a total of 12,000 μg (12.0 mg). The median effective dose was 1,000 to 1,250 μg for a 20 g mouse, or 50 to 62 μg/g when one intraperitoneal injection was given on the 48th hour of infection. Bacteriological autopsies demonstrated that on the 14th hour after injection of the antibiotic,

* This work, recommended by the Committee on Medical Research, was done in part under a contract between the Office of Scientific Research and Development and the University of California.

1 Girard, *Bull. office int. d'hyg. publ.*, 1936, **28**, 1078.

2 Jawetz and Meyer, *J. Infect. Dis.*, 1943, **73**, 124.

3 Otten, *Indian J. Med. Res.*, 1936, **24**, 73.

the spleens of 4 mice sacrificed were sterile; however, plague bacilli could be cultured from the heart blood of 2/4, the livers of 3/4, and the lymph nodes of 4/4. Organs of control mice showed heavy growth of *P. pestis* in cultures and microscopically. Begun on the 48th hour of infection, 100 μg of streptomycin injected at 6-hour intervals for 192 hours, or 32 injections in all (a total of 3.2 mg of streptomycin per mouse), always sterilize the blood stream, spleen and liver, but since the infection persists in the lymph nodes, about 40% of the animals ultimately succumb. The rate of survival in mice is directly proportional to the amount of streptomycin administered. A large dose of antibiotic at the outset of infection probably kills or injures the bacillus, inhibits its multiplication and enhances receptivity to phagocytosis, which effects its removal from the circulation. There is a possibility that the regional bubo, with its abundant necrotic tissue and large number of plague bacilli, is not immediately affected, and thus serves as a seed-bed for relapses and a continuous source of toxin when the immunity mechanism is not completely mobilized. The larger the dose, the greater the possibility for streptomycin to diffuse into necrotic areas, and the shorter the course of therapy needed. In the mouse the greatest dose, or that determined by toxicity, is more than ten times the effective dose that was used in the treatment of plague infections.

Bubonic plague infections which have progressed to an extent comparable to 48-hour infections in mice occur in guinea pigs 120 hours after subcutaneous injections of 1,000 multiples of the M.L.D. With a dose of streptomycin of 20,000 μg/kg or approximately 10 mg per day, a total of 100 mg over a period of 10 days, 80 to 100% of the 40 guinea pigs used in experiments were cured. It requires less streptomycin to cure guinea pigs in a stage of mild septicemia than to cure mice, on the basis of units of antibiotic to grams of body weight. The administration of 25,000 to 50,000 μg/kg in 3 doses, or a daily injection of 37,500 to 75,000 μg/500 g guinea pig of the early streptomycin preparations proved toxic, but recent lots of the antibiotic in the same doses are well tolerated.

In the tests under consideration and those of Wayson and McMahon[4] persistence of plague bacilli in regional buboes despite large doses of the antibiotic suggested local chemotherapy. Guinea pigs with well-developed buboes on the 120th hour after subcutaneous administration of 100,000 *P. pestis,* were injected around the local swelling with 12,500 μg in 0.5 cc of physiologic saline at 12-hour intervals for 10 days. All were cured and plague lesions were sterilized. Sodium sulfamerazine administered in the same manner in the amount of 500 mg/kg/12 hours was inhibitory but not bactericidal, and cured no more than 40% of the infected animals. Local injections of streptomycin proved very irritating, and even though buboes became rapidly sterile, the necrotic areas were much larger than those of controls or of animals treated systemically.

(3) *Effect on septicemic plague in mice.* To establish the maximum effectiveness of streptomycin, a rapidly progressive *septicemic* infection was produced in slightly-resistant Swiss mice by intraperitoneally injecting 2,000 *P. pestis.* Bacteremia and toxemia exist in such mice by the 9th-12th hour after infection, and death takes place in 34 to 72 hours. In some respects, this infection model resembles the clinical form of human septicemic plague induced by direct blood stream infection through several fleabites. Repeated tests of the kind shown in Fig. 1 conclusively demonstrated that 2 to 3 large doses of 400 to 800 μg at 3-hour intervals, or a total of 1,200 to 1,600 μg, cured 80 to 90% of non-bubonic septicemic plague infections. Small doses merely delay death. If treatment is postponed until the 18th or 24th hour, 2 doses of 800 μg each cure only 25 to 45% of infected mice. Only early treatment with large doses of streptomycin cures septicemic plague in mice. Sulfadiazine and highly potent antiplague serum proved ineffective under

[4] Wayson and McMahon, *J. Lab. and Clin. Med.,* 1946, **31**, 323.

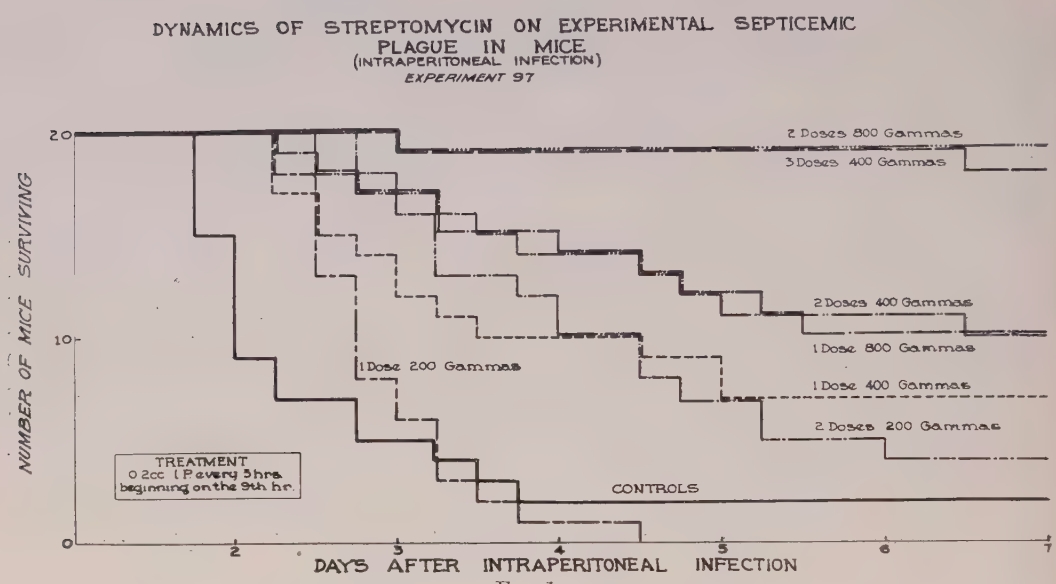

FIG. 1.

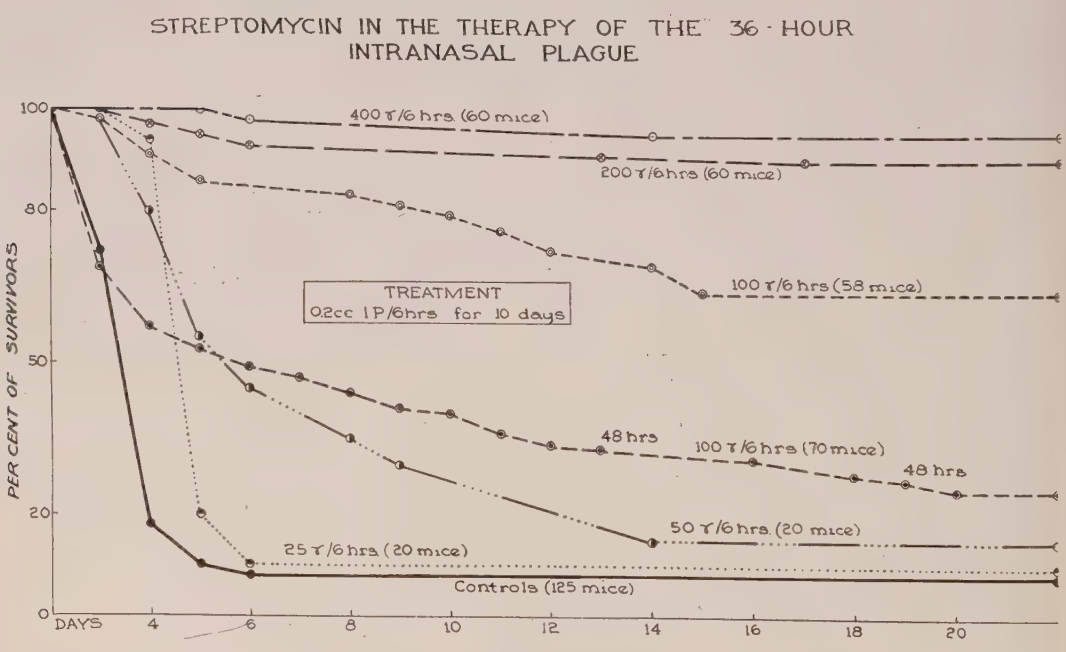

FIG. 2.

identical experimental conditions. There is at present no evidence that sulfonamides synergistically enhance the remedial action of streptomycin. On the other hand, numerous experiments furnished data that potent antiplague serum administered simultaneously with small doses of streptomycin increased the percentage of cures. Finally, active immunization with plague antigens potentiates the action of streptomycin both in mice and in guinea pigs and the partial immunity so gained allows use of smaller doses of streptomycin.

(4) *Effect on pneumonic plague in mice.* Pneumonic plague infections may be produced in mice by intranasal instillation of 2,500-5,000 *P. pestis* in 0.05 cc of saline; the lobular lesions recognizable on the 36th hour after infection contain several million

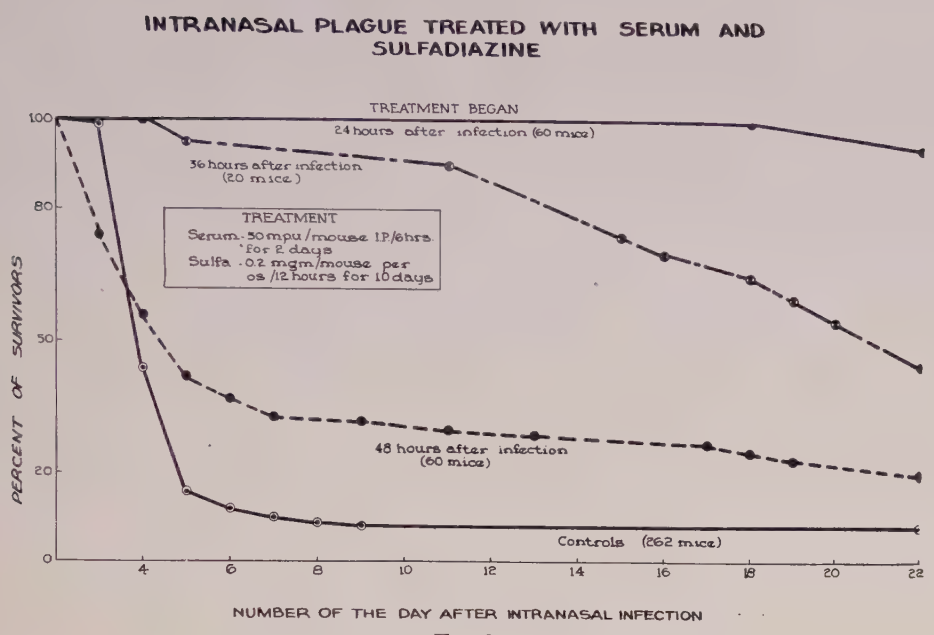
INTRANASAL PLAGUE TREATED WITH SERUM AND SULFADIAZINE
TREATMENT BEGAN
24 hours after infection (60 mice)
36 hours after infection (20 mice)
TREATMENT
Serum - 30 mpu/mouse IP/6hrs. for 2 days
Sulfa - 0.2 mgm/mouse per os/12 hours for 10 days
48 hours after infection (60 mice)
Controls (262 mice)
PERCENT OF SURVIVORS
NUMBER OF THE DAY AFTER INTRANASAL INFECTION

FIG. 3.

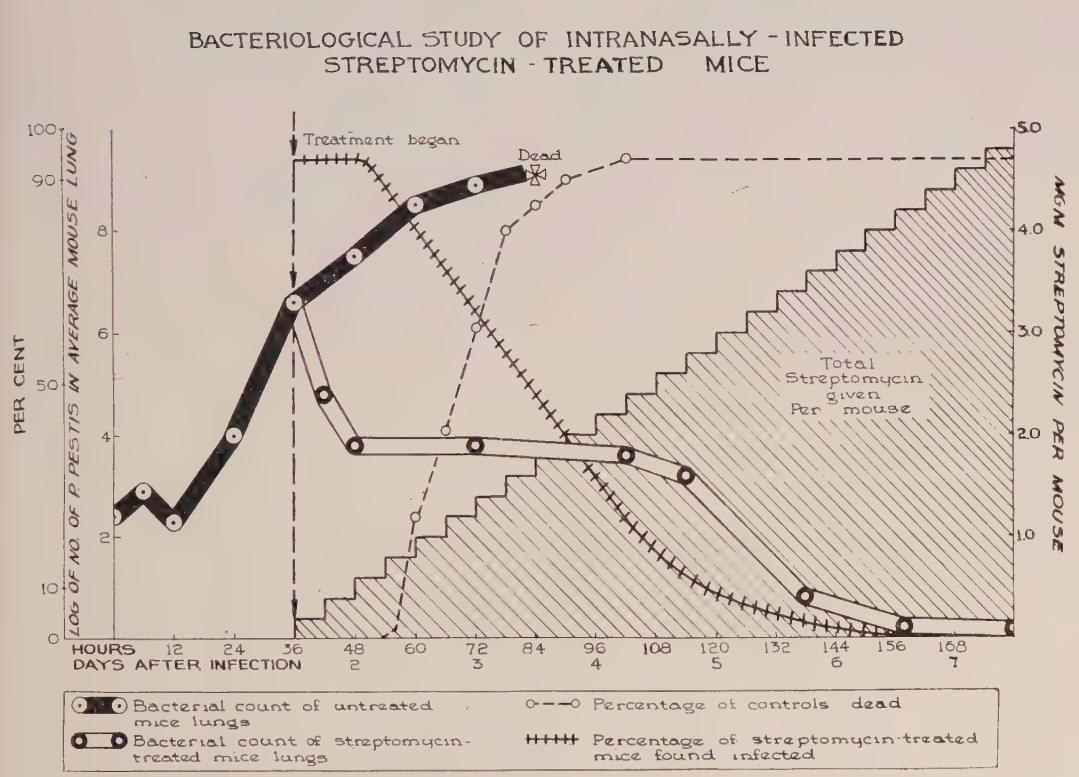
BACTERIOLOGICAL STUDY OF INTRANASALLY - INFECTED STREPTOMYCIN - TREATED MICE
Treatment began
Dead
Total Streptomycin given Per mouse
PER CENT
LOG OF NO. OF P. PESTIS IN AVERAGE MOUSE LUNG
MGM STREPTOMYCIN PER MOUSE
HOURS
DAYS AFTER INFECTION
Bacterial count of untreated mice lungs
Bacterial count of streptomycin-treated mice lungs
Percentage of controls dead
Percentage of streptomycin-treated mice found infected

FIG. 4.

plague bacilli. As might be expected, 200 to 400 μg of streptomycin hydrochloride or sulfate, given every 6 hours, effectively cure 90 to 95% of the infections. Smaller doses or delayed treatment reduces the chance for cures (Fig. 2). Sulfadiazine in combination with antiplague serum is less effective than streptomycin (Fig. 3). The remarkable bactericidal action of streptomycin is fully documented by periodic bacteriological autopsies of treated and untreated mice (Fig. 4). Six hours after treatment with 200 μg had begun, the number of plague bacilli found in the lungs of the treated mice was reduced to approximately 60,000, while in untreated animals it had advanced to 10,000,000. By the 12th to 24th hours of treatment, the spleen became sterile and 5000 or less organisms were counted in the lungs. By the 96th hour after infection, when all untreated mice had died, the lungs and bronchial lymph nodes of treated mice were either sterile or contained only a few thousand plague bacilli in the abscess-like patches of pneumonia. No plague bacilli have been isolated from lungs or lymph nodes 100 hours after treatment with streptomycin. These results fully attest to the remarkable therapeutic efficacy of a total of 5 mg of streptomycin in experimental plague of mice. They justify an expectation that it will be equally effective in human pneumonic plague if administered early and in adequate dosage.

(5) *Suggested schedule of treatment in human plague.* Streptomycin is the most effective therapeutic agent thus far discovered for the treatment of bubonic, septicemic and pneumonic experimental plague infections in mice and guinea pigs. It is recommended that human plague be treated as soon as diagnosed with daily doses of 2 g of streptomycin in bubonic plague, and 4 to 6 g in the septicemic and pneumonic diseases; injections should be given at 4-6 hour intervals for the first 2 days. The dose may then be reduced, but in order to prevent clinical recurrences treatment should be continued for at least 8 days on a one g level or substituted with adequate sulfadiazine therapy. In profound toxemia, simultaneous administration of a potent antiplague serum, to assist the immunity mechanism, may prove beneficial.

Summary. Streptomycin in the amounts of 0.4 to 4.0 μg/cc is bactericidal for different strains of *P. pestis* in 5 days. Advanced experimental bubonic plague in mice is completely cured with 500 μg/3 hours for 3 days, or a total of 120 mg. Between 80 to 90% of the mice in a state of septicemic plague may be saved with a total of 1,200 to 1,600 μg. The remarkable bactericidal action of streptomycin is best demonstrated on experimental pneumonic plague; 5 mg of the antibiotic sterilize lungs and lymph nodes within 100 hours after treatment has been instituted. It is recommended that human plague be treated as soon as diagnosed with 2 to 4 g of streptomycin daily depending on the state of infection.

16147

Distribution of P^{32} in Incubated Egg.*

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As part of our observations of incubated eggs injected with P^{32} we traced the course of the isotope within the components of the egg throughout the incubation period. The phosphate content of a hen's egg of this size is approximately 220 mg[1] and the amount

* Work done under contract from Office of Naval Research.

of phosphate injected in this procedure was 3.5 mg.

Materials. Eggs—White Leghorns average weight 60 g incubated at 38°C with 70-80% relative humidity. P^{32} as KH_2PO_4—a 10% solution of the salt in H_2O was used for injection. The P^{32} was supplied as KH_2PO_4 by Clinton Laboratories, U. S. Government, Oak Ridge, Tenn.

Procedure. Approximately 0.05 cc of 10% KH_2PO_4 solution containing 20 μc per 0.05 cc was injected into the yolks of fertile eggs on the 5th day of incubation. One or 2 of these eggs were analysed daily during the remaining 15 days of incubation to determine the location and chemical combination of their P^{32}.

The analysis consisted of first separating the embryo, allantoic and amniotic fluid, yolk, albumen, and shell. Because of technical difficulties the allantoic and amniotic fluids were used together and not separated. The fluids were measured with a pipette or a tuberculin syringe and estimated to the nearest 0.5 cc. Greater accuracy in determination of these fluid volumes was not possible because of some mixing of the fluids during separation. The embryo and shell were weighed. Second, the radioactivity of these various components was measured with a Geiger counter. In order to avoid any errors due to selfabsorption, the embryos were completely ashed before measurement; the shells were dissolved in HCl and an aliquot of this solution was used for measurement. Third, in the eggs from the 6th to the 14th day of incubation each of the three fluid components was divided into ether extract, trichloracetic acid precipitate, and water soluble fraction after precipitation with trichloracetic acid. This was done by mixing 2 cc of the fluid sample with 8 cc of H_2O and extracting with ether for 15 minutes. To the H_2O soluble fraction was added 5 cc of 20% trichloracetic acid. This was filtered. After evaporation the activity of each of these fractions was measured.

Results. Complete results are listed on the accompanying table. Within 24 hours after injection into the yolk, ⅔ of the P^{32} had left the yolk and was found principally in the allantois and amnion. After the first day, the balance of labeled phosphate in the yolk remained constant for 4 or 5 days and then gradually diminished in amount until at hatching time about 5% remained in the yolk. During this period the volume of yolk had diminished to about half of its original volume.

Within 24 hours after injection, more than ½ of the P^{32} was in the allantoic and amniotic fluids. Subsequently, the amount of radioisotope in these fluids decreased in proportion to the uptake by the embryo, until at hatching time they contained only 3-4%. The volume of these fluids was constant until the last 4 days of incubation when it decreased rapidly.

The embryo's content of P^{32} increased nearly in proportion to its gain in weight. Shortly after injection its concentration was about 1.5 μc/g of embryo, and this ratio was maintained until the last 6 days when the concentration was closer to one μc/g of embryo. At hatching time the embryo contained about 90% of the P^{32} and made up a little more than ½ the total weight of the egg.

The albumen never contained more than 3% of the injected radio-isotope. As incubation continued, the drop in P^{32} more or less paralleled the decrease in volume of albumen.

The shell and its membrane contained only 1% of the P^{32} throughout incubation. This small amount of activity lies within the experimental error because of the obvious difficulty of separating shell from all fluid contents.

Thus it is apparent that within 24 hours following injection, a shift of P^{32} from yolk to allantois and amnion takes place. As the embryo grows, its rate of uptake of the phosphate from allantois and amnion is much greater than from the yolk.

In addition to the above analysis we determined for each fluid constituent the amount of P^{32} contained in the H_2O soluble fraction, the ether soluble fraction and the protein precipitate. The distribution of P^{32} within

[1] Needham, J., *Chemical Embryology*, Vol. II, p. 1199, Cambridge University Press, London, 1931.

TABLE I.
Distribution of P^{32} in Incubated Eggs.
(Approximately 5 mg KH_2PO_4 containing 20μc P^{32} injected into yolk 5th day of incubation.)

Age of egg	Embryo		Allantois and amnion		Yolk		Albumen		Shell and membrane.
	Wt in g	% of total activity	Volume in cc	% of total activity	Volume in cc	% of total activity	Volume or wt	% of total activity	% of total activity
6	0.4	3	13.5	64	20	29	17 cc	2	2
7	0.8	6	14	52	20	37	14 "	2	1
8	1.4	10	13	53	22	36	12 "	1	1
9	2.2	18	15	50	21	29	11 "	3	1
10	3.1	28	11	29	26	39	10 "	3	1
11									
12	5.8	58	14	21	14	19	8 "	1	1
13	8.1	65	10.5	12	16	19	11.5 "	3	1
14	9.3	71	13	12	20	17	7 "	.7	1
15	13.3	73	13.5	13	14	12	5 "	.8	1
16	16.9	73	11	16	14	7	3 "	.6	1
17	18.1	76	6.5	17	14	7	1 g		.8
18									
19	25.5	93	3	3	14	4	1 "	.3	.3
20	27.4	90	7	4	9	5	.5 "	.4	.6

these various fractions was relatively constant throughout our period of observation from the 6th to the 14th day of incubation. Ninety per cent of the P^{32} in the allantoic and amniotic fluids was found in the H_2O soluble fraction, 9.4% in the trichloracetic acid precipitate and 0.6% in the ether soluble extract.

In the yolk nearly all of the activity was in the H_2O soluble fraction, only 1.1% was found in the ether extract.

In the albumen 80% of activity was found in H_2O soluble fraction and 20% in trichloracetic acid precipitate.

Summary. The radioactive isotope P^{32} was used as a tracer to follow the course of phosphorus combined as KH_2PO_4 and injected into the yolks of incubating eggs. The distribution of P^{32} within the various components of the eggs was determined daily throughout incubation. The distribution in ether soluble, water soluble, and trichloracetic acid precipitable fractions was determined for the fluid components of the eggs from the 6th to the 14th day of incubation. From the 6th day of incubation to the day of hatching, the P^{32} in the embryo increased from 3% to 90%.

16148

Changes in Plasma Inorganic Phosphorus of Dogs Under Postural Restraint.*

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During the course of some routine experiments the observation was made that, in unanesthetized dogs restrained in the supine position, the inorganic phosphorus concentration of whole blood and of plasma decreased to a low value of 30-60% of the original amount in 2 hours, after which it increased, in some instances attaining a level about

* Aided by a grant to Vanderbilt University, School of Medicine, from the Bristol-Myers Company.

† The author wishes to express his indebtedness to Dr. Gladys R. Bucher for much helpful advice and criticism.

TABLE I.
Average Plasma Inorganic Phosphorus Values in Dogs Restrained in the Supine Position.

Duration of restraint (min.)	Dog 1 (4 exp.)			Mean of 4 dogs (17 exp.)		Mean of all samples		
	M	S	A	M	A	M	S	A
0-9	4.36	0.34	100.	4.05	100.	4.07	1.10	100.
10-29	3.51	0.30	81.	3.52	87.	3.45	0.94	85.
30-59	2.38	0.35	55.	3.45	85.	2.58	0.62	63.
60-119	2.26	0.55	52.	2.23	61.	2.22	0.80	55.
120-239	2.55	0.94	58.	2.46	67.	2.23	1.20	55.

M Mean values in mg %.

S Standard deviation; S equals $\sqrt{\frac{\Sigma(X-M)^2}{n-1}}$

A Per cent of initial concentration.

equal to the original value.

Blood phosphate changes in normal animals have frequently been observed upon administration of substances such as insulin or epinephrine[1,2] or upon the ingestion of glucose,[3] but we have found in the literature no report of changes in blood phosphate in animals which, other than being restrained on their backs, were allowed to remain under normal conditions.

In view of these facts and since the phosphate changes we had observed were so marked and consistent, we investigated further the occurrence of this phenomenon under two different conditions of postural restraint: first, that in which the animals were tied in the supine position for 2 to 4 hours and second, that in which they stood in a Pavlov stock for the same period of time.

Methods. In the first group of experiments blood samples were drawn from the femoral vein immediately after the dogs were laid on the table and again at 30, 60, 120, and 240 minutes later. In the second group the animals were placed in a Pavlov stock after a "control" blood sample was taken.

Immediately after being drawn the blood was delivered into cold centrifuge tubes containing dried sodium oxalate and surrounded by crushed ice, centrifuged, and the plasma kept chilled until the inorganic phosphorus analysis was completed.

Inorganic phosphorus was determined by the method of Lowry and Lopez[4] on the ice-cold trichloracetic acid filtrate of the chilled plasma. Total phosphorus, acid soluble phosphorus, and lipid phosphorus, (after extraction with alcohol-ether mixture) all were evaluated after sulfuric-nitric acid digestion (2.5 ml of 5 N sulfuric acid and 3 drops of concentrated nitric acid) by addition of 2 ml of 2.5% ammonium molybdate solution and 3 ml of 1% fresh ascorbic acid solution. Exactly 20 minutes after addition of the ascorbic acid the developed color was measured in a Klett-Summerson photoelectric colorimeter, using filter No. 66. Blanks and standards were always done parallel to unknowns and duplicate samples were analyzed whenever possible.

Plasma glucose was estimated by a modification of the Somogyi method reported by Nelson.[5]

Results and Discussion. Marked decreases in plasma inorganic phosphate averaging 52% of the initial level were observed in all but one of a total of 17 experiments done on 4 dogs in the first group. Table I shows the mean values of all 17 experiments as well as average results of 4 experiments done on Dog 1 as a representative example. The mean of

1 Perlzweig, W. A., Latham, Emily, and Keefer, C. S., PROC. SOC. EXP. BIOL. AND MED., 1923, **21**, 33.

2 Harrop, George A., Jr., and Benedict, E. M., *ibid.*, 1923, **20**, 430.

3 Harrop, George A., Jr., and Benedict, E. M., *J. Biol. Chem.*, 1924, **59**, 683.

4 Lowry, Oliver H., and Lopez, Jeanne A., *ibid.*, 1946, **162**, 421.

5 Nelson, Norton, *ibid.*, 1944, **153**, 375.

TABLE II.
Plasma Phosphorus Fractions and Glucose Content, Dog 4, Exp. 17.

Duration of restraint (min.)	Phosphorus (mg %)				Glucose (mg %)	% of initial conc.	
	TP	LP	TASP	IP		IP	Glucose
0*	14.9	11.1	3.28	3.04	98.4		
0-9	14.5	11.3	3.34	3.18	107.	100.	100.
10-29	—	—	—	—	—	—	—
30-59	13.8	11.3	2.76	2.68	107.	84.	100.
60-119	12.5	11.1	0.93	0.97	158.	31.	151.
120-239	12.3	11.1	1.24	1.04	158.	33.	151.

* This blood taken 60 minutes prior to restraining the dog.
TP Total Phosphorus.
LP Lipid Phosphorus.
TASP Total Acid Soluble Phosphorus.
IP Inorganic Phosphorus.

TABLE III.
Plasma Inorganic Phosphorus Concentration of Dogs Restrained in a Stock.

Duration of restraint (min.)	Inorganic Phosphorus (mg %)		% of initial conc.
	Dog 3	Mean of 4 dogs	
0	4.76	4.64	100.
1-29	4.28	4.08	88.
30-59	4.28	3.79	82.
60-119	4.28	4.04	87.
120-239	4.48	3.98	86.

all the samples, also included in this table, indicates significant decreases through the 60-119-minute period, after which the concentration remained constant or increased slightly in most of the dogs.

In 8 of the 17 experiments performed with the dogs restrained in the supine position a complete analysis of the plasma phosphorus fractions was done, and in 3 of these the plasma glucose levels were also followed. A complete analysis of one of these three experiments is presented in Table II. In this experiment the dog was allowed to remain unrestrained on the floor for about one hour and then tied in the supine position for 2 to 4 hours. The dog was quiet while on the floor but showed signs of unrest soon after being confined. The irritability increased markedly after the 60-minute period and continued until the animal was set free. In all 8 experiments the acid soluble and total phosphorus paralleled the changes in the inorganic fraction, while lipid phosphorus remained quite constant during an entire experiment. In general, increases in plasma glucose were observed at about the same time or immediately after decreases in plasma inorganic phosphorus were noted.

Our animals, although trained to lie quietly on their backs, at times exhibited signs of emotional disturbances and fatigue. A study of the decreases in the obviously excited animals and in the apparently quiet ones revealed an average low value of 40% of the original concentration in the former group compared to 69% for the latter. This difference, when studied statistically, is not significant, the probability at the 60-119-minute period being 17%. Analysis of a larger number of experiments might show a more significant difference between the 2 groups.

The supine position is not a natural one for the dog and restraint in such a position might possibly be the cause of physiological changes in the animal, either directly because of the peculiar posture or indirectly because of aroused emotional status due to fatigue. In 4 experiments dogs restrained in a standing position in a stock did not offer any resistance or become excited to any noticeable

extent. In these experiments, the results of which are presented in Table III, there was a slight decrease in plasma inorganic phosphate, the greatest decrease occurring at the 30-59-minute period and amounting to 13% of the initial value.

It is not possible from the data obtained to derive any conclusions concerning the exact cause of these changes occurring in the plasma phosphorus concentration of restrained dogs. The principal factors appear to be those of posture and of emotional disturbance. This paper does not attempt to establish the relative contribution of each of these factors to the phosphorus decrease; its purpose is to report the observation of this phenomenon so as to bring it to the attention of those investigators whose experiments may be affected by its occurrence.

Summary. 1. A decrease in the plasma inorganic phosphate concentration amounting to 30-60% of the initial concentration was observed in dogs restrained in the supine position. This change was also evident in the total acid soluble and the total phosphorus fractions, while the lipid phosphorus fraction remained constant during an entire experiment.

2. The decrease in inorganic phosphate was accompanied by an increase in plasma glucose.

3. Dogs restrained on their backs exhibited a greater decrease than those restrained in a stock.

16149

Effect of Salicylate and Tripelennamine Hydrochloride (Pyribenzamine) on the Arthus Reaction and on Bacterial Allergic Reactions.*

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The tissue damage that results from the union of certain antigens with their corresponding antibodies may be prevented by non-specific agents.[1,2] In the case of the positive tuberculin reaction, cachexia and fever appear to inhibit the expected reactions.[2] As yet, however, little attention has been directed to the inhibition of necrotizing allergic reactions by therapeutic agents. The more transient reactions of classical anaphylaxis are found to be prevented effectively by the Mosnier compounds which have striking anti-histamine properties.[3,4] Urticarial reactions to specific allergens or to histamine can be inhibited by many of these synthetic compounds, of which the most widely used in this country are diphenhydramine hydrochloride (Benadryl) and tripelennamine hydrochloride (Pyribenzamine). Clinically, hayfever and analogous reactions have been benefitted by these agents.[4] These substances, however, have not been demonstrated to be beneficial in the "bacterial" type of allergic response, or in the local Arthus reaction, although these allergies may be important in the pathogenesis of many diseases. Reubi[5] reported that 2-(N-phenyl-N-benzyl-aminoethyl)-imidazoline (antistine) prevented the occurrence of the kidney damage that usually results when duck anti-kidney serum is in-

* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

1 Mitchell, A. G., Wherry, W. B., Eddy, B., and Stevenson, F. E., *A. J. Dis. Child.*, 1928, **36**, 720.

2 Gay, F. P., and Associates, *Agents of Disease and Host Resistance,* Springfield, Charles C. Thomas, 1935.

3 Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 703.

4 Friedlander, S., *Am. J. Med.*, 1946, **1**, 174.

5 Reubi, F., *Helvetia Medica Acta*, 1946, **13**, Supp. XVIII.

jected into rabbits. Attempts to use similar compounds such as diphenhydramine or tripelennamine to inhibit the tuberculin reaction in animals were unsuccessful.[6] Likewise the use of these drugs in two cases of serum sickness and 3 of rheumatic fever has been disappointing in our experience.

Among the agents said to inhibit the more severe allergic tissue reactions, salicylates have been frequently mentioned. Derick, *et al.*[7] used aspirin to prevent the appearance of some of the manifestations of serum sickness. Several workers have presented data which led them to believe that salicylates inhibited antibody formation.[7–10] Coburn demonstrated that concentrations of sodium salicylate larger than those usually obtained clinically inhibited the union of antigen and antibody *in vitro*,[11] and on this basis used salicylates prophylactically to prevent recurrences of rheumatic fever following streptococcus infections.[12]

It appeared of interest to study whether, in the presence of known quantities of antibody and antigen,[16] salicylate or tripelennamine could inhibit the Arthus reaction induced passively in rabbits. The "bacterial" type of allergic reaction in humans actively sensitive to tuberculin or to streptococcal nucleoprotein was also studied.

The Arthus Reaction. The desirability of inducing allergic reactions with known amounts of a single pure antigen and its corresponding antibody is obvious from the data of Kabat.[13] He and his coworkers[14–16] have presented the techniques for producing anaphylaxis and the Arthus reaction with measured amounts of antigen and antibody. The latter technique was employed in this study.

Albino rabbits (about 2200 g) were injected intracutaneously in single or multiple sites with dilutions of rabbit anti-chicken egg albumin serum containing amounts of antibody nitrogen known to give reactions of minimal and maximal severity.[16] The serum was prepared and analyzed by Dr. E. A. Kabat and kindly given for this study. Controls were injected in equal number simultaneously but were not given drug therapy. After a half hour, known amounts of a solution of 4 times recrystallized egg albumin were injected directly into the sites. The reactions were read at 6, 12, 24 and 48 hours, and those of the untreated animals were compared with groups receiving salicylate or tripelennamine. In some instances biopsies were taken of the involved areas for histological comparison. The intensity of the reactions was graded as previously described:[16] 0 = no reaction; ± = transitory erythema lasting 5 to 8 hours, and absent at 24 hours; + = erythema lasting 24 hours and measuring up to 1.5 cm in greatest diameter at that time; ++ erythema 1.5-2.5 cm at 24 hours persisting for one to 2 days, with slight edema and occasional slight brownish discoloration after 24 hours; +++ 2-3.5 cm erythema with moderate edema and slight brownish discoloration persisting 2 to 3 days; ++++ more than 2.5 cm of erythema, marked edema and moderate or marked brownish discoloration with changes lasting 3 to 5 days. Rabbits of the treated groups were given the medication every 4 hours on the day before, the day of, and occasionally on the day following the induction of the Arthus reaction, although by this time the reactions had reached their height and readings were conclusive. Sodium salicylate was given intravenously in doses of 2 to 5 ml of a 10%

6 Birkeland, M., and Kornfeld, L., *J. Bact.*, 1947, **54**, 82.

7 Derick, C. L., Hitchcock, C. H., and Swift, H. F., *J. Clin. Invest.*, 1928, **5**, 427.

8 Swift, H. F., *J. Exp. Med.*, 1922, **36**, 735.

9 Homburger, F., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 101.

10 Jager, B. V., and Nickerson, M., *Am. J. Med.*, 1947, **3**, 408.

11 Coburn, A. F., and Kapp, E. M., *J. Exp. Med.*, 1943, **77**, 173.

12 Coburn, A. F., and Moore, L. V., *J. Pediat.*, 1942, **21**, 180.

13 Kabat, E. A., *Am. J. Med.*, 1947, **3**, 535.

14 Kabat, E. A., and Landow, H., *J. Immunol.*, 1942, **44**, 69.

15 Kabat, E. A., and Boldt, M. H., *J. Immunol.*, 1944, **48**, 181.

16 Fischel, E. E., and Kabat, E. A., *J. Immunol.*, 1947, **55**, 337.

TABLE I.
Effect of Salicylate and Tripelannamine on Severity of the Arthus Reaction Induced Passively with Known Amounts of Anti-egg Albumin and Crystalline Egg Albumin. Number of Reactions of Different Severity Related to Total Reactions in Each Group.

Antibody nitrogen (mg)	Treatment	Severity of reaction with 0.13 mg crystalline egg albumin nitrogen				
		++++	+++	++	+	0
.53	Control	3/3				
	Salicylate	2/3	1/3			
.22	Control	3/16	9/16	4/16		
	Salicylate	1/8	5/8	2/8		
	Tripelennamine		4/10	6/10		
.10	Control			1/2	1/2	
	Tripelennamine				2/3	1/3
.05	Control			4/7	3/7	
	Salicylate			4/7	2/7	1/7
	Tripelennamine			4/5	1/5	
.025	Control				2/2	
	Tripelennamine				1/3	2/3

solution (0.2 to 0.5 g). In a few instances serum salicylate levels were determined.[17] A value of 1224 γ per ml was found a few minutes after one injection of 0.4 g and 311 γ per ml after 4 hours. Tripelennamine hydrochloride (Pyribenzamine) was administered as an 0.5% solution in doses from 20 mg intramuscularly to 5 mg intravenously on the day before and at 4-hour intervals on the day of induction of the Arthus reaction. Ten mg intravenously caused transient convulsions in some rabbits.

The results of the systemic administration of salicylate or Pyribenzamine on the severity of the passive Arthus reaction are presented in Table I. It is apparent that the severity of the reaction was not appreciably altered when the higher concentrations of antibody were used, or with quantities normally giving a minimal reaction. The readings tabulated are those at the end of 24 hours, in conformity with our previous experience, and with the generally accepted picture of the Arthus phenomenon. However, during the first 6 or 8 hours a difference between the control and salicylate treated groups was noted which does not lend itself to adequate measurement. Usually the control and tripelennamine treated groups had a thicker and slightly wider area of edematous skin than did the salicylate treated group. The reaction at 24 hours more closely approximates the tissue damage associated with the classical Arthus phenomenon and apparently the end reaction is not influenced by the moderate difference in edema at the end of 6 hours.

Bacterial Allergic Reaction. "Bacterial" allergic reactions have been distinguished from the Arthus reaction chiefly because it is not possible to transfer the allergy to bacterial products which results after infection with serum of sensitized individuals.[18] Chase[19] showed that sensitivity of the tuberculin type may be passively transferred with cellular exudates from sensitized animals. The mechanism of this cellular transfer of sensitivity is not yet clearly understood. In these studies it was necessary to determine the initial degree of sensitivity and then subject the same individual to the salicylate or tripelennamine and ascertain the effect on the reaction.

Subjects were adults on the wards of the Presbyterian Hospital recovering from vari-

[17] Brodie, B. B., Undenfriend, S., and Coburn, A. F., *J. Pharm. and Exp. Therap.*, 1944, **80**, 114.

[18] Rich, A. R., *The Pathogenesis of Tuberculosis*, Springfield, Charles C. Thomas, 1941.

[19] Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 134.

TABLE II.
Effect of Salicylate and Tripelennamine on Intracutaneous Skin Tests in Individuals Sensitive to Streptococcal Nucleoprotein Fraction C18K.

Subject	Area of erythema			
	Control (inches²)	Salicylate (inches²)	Salicylate level (γ/ml)	Tripelennamine (inches²)
R.A.	.15	.20	321	.23
E.C.	.33	.31	548	.53
L.C.	.30	.50	352	.39
B.C.	.11	.19	534 (toxic)	.17
B.D.	.30	.42		.28
H.H.	.35	.32	278	.58
Y.M.	.05	.14	338	
M.R.	.16	.26		.25
A.S.	.50	.58		.60
P.W.	.40	.10	507 (toxic)	.60
D.	.27	.40		
X.C.	.19	.05		
Y.C.	.07	.12		
I.W.	—	.37		.50

ous diseases, medical students, and children convalescing from rheumatic fever at the Pelham Home for Children.† Intracutaneous skin tests were done on comparable areas of the forearm but individual sites were used only once. The purified protein derivative (PPD) of tuberculin[20] and a nucleoprotein fraction C18K prepared from the hemolytic streptococcus by Heidelberger and Kendall[21] and kindly given for this study by Dr. Heidelberger, were used as antigens. The PPD was used in one of the 2 customary strengths, 0.00002 mg in 0.1 ml and 0.005 mg in 0.1 ml. The C18K was diluted from an analyzed (Kjeldahl) stock solution so that 0.1 ml contained 0.0001 mg nitrogen. Readings were made as frequently as possible, every 6 or 12 hours after injection. The C18K skin test appeared to be at its maximum at 24 hours in all cases except the most severe reaction which went on to become hemorrhagic at 48 hours. The other cases showed well demarcated areas of erythema and edema or induration, occasionally surrounded by a zone of erythema alone. In instances where the zone was present, the areas of both ovals were determined, but only the outer one is listed in Table II. Skin reactions were recorded by cellophane tracings of the lesions and an appraisal of the degree of erythema and induration was made to compare further the reactions in the same individual. Medication was given orally, either a week before or a week after a control test was performed. Salicylate was given as enteric coated aspirin or sodium salicylate in doses of 0.6 to 1.2 g every 4 hours, night and day, giving salicylate levels from 153 γ/ml to 534 γ/ml in a representative group of subjects. Tripelennamine (Pyribenzamine) was given in doses of 25 to 50 mg 3 times a day for a few days before and during the skin reacting period.

Tests were done 2 or 3 times in the same individual, one time without any medication and the other one or 2 times at weekly intervals with salicylate or tripelennamine administered. The results of the C18K skin tests are seen in Table II, which lists the area of the cellophane tracings of the skin lesions as determined by planimeter. Other characteristics of the lesions such as degree of erythema and induration are not listed since they did not vary appreciably from test to test in the same individual.

Thirteen subjects were tested with C18K with and without salicylate administration.

† We are grateful to Miss Mary C. Kelly and the Pelham Home for Children for their cooperation.

20 Seibert, F. B., Aronson, J. D., Reichel, J., Clark, L. T., and Long, E. R., *Am. Rev. Tuberc.*, 1934, **30**, Supp.

21 Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1931, **54**, 515.

Of these only 2 showed diminution of the area of the skin reaction of more than 0.1 square inch during aspirin administration, one of these had marked signs of salicylism and a level of 507 γ/ml. Seven individuals had no difference in area with and without salicylates and 4 had increases of more than 0.1 square inch despite comparable doses of salicylate. With tripelennamine, the C18K reaction was approximately the same as the control reaction in 5 individuals and increased 0.1 square inch or more in 4 individuals. A similar degree of variation is present when the reactions with salicylate are compared with those during tripelennamine administration. Four individuals did not show any change, 2 had lesions which diminished by more than 0.1 square inch and four showed areas which increased by more than 0.1 square inch. With the tuberculin test, 22 subjects were given aspirin; 14 showed no appreciable change from their control skin test, 5 showed a more severe reaction, and three had a less severe reaction.

Discussion. The Arthus reaction is a manifestation of the union of certain antigens with antibodies, and its severity can be related to the amount of antibody uniting with the antigen.[16,22,23,24] Since the severity of the Arthus reaction was not altered appreciably it does not appear that the drugs employed inhibit the union of antigen with antibody *in vivo*. However, the moderately diminished edema at the end of 6 hours in the salicylate treated group may be of interest in view of the demonstrated inhibition by salicylate of spreading due to hyaluronidase.[25]

Tripelennamine has been shown to exhibit a significant antihistamine action.[3,4] Since it did not lessen the severity of either lesion studied, it appears that histamine does not play a significant role in the pathogenesis of necrotizing allergic reactions.

Summary. A study was made of the effect of salicylate and of a synthetic antihistamine compound, tripelennamine hydrochloride, on necrotizing allergic reactions of the Arthus and "bacterial" types. The Arthus reaction was induced passively in rabbits by quantitative methods. "Bacterial" sensitivity to a streptococcus nucleoprotein fraction and to tuberculin was observed in human subjects. Neither type of reaction appeared to be altered by the administration of salicylate or tripelennamine. However, in the early development of the Arthus phenomenon, salicylate treated animals exhibited less edema at the site of the lesion.

22 Opie, E. L., *J. Immunol.*, 1924, **9**, 231.

23 Culbertson, J. T., *J. Immunol.*, 1935, **29**, 29.

24 Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1941, **40**, 127.

25 Guerra, F., *J. Pharm. and Exp. Therap.*, 1946, **87**, 193.

16150

Further Observations on the Cultivation of Strains of Poliomyelitis Virus in Developing Eggs.*

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In an earlier paper[1] we reported the successful cultivation of the murine SK strain in developing eggs. In the present paper we wish to summarize observations made since then on this and other strains.

Murine SK Strain. The SK strain has now

* Supported by the Howard Frost Poliomyelitis Research Fund.

1 Schultz, E. W., and Enright, J. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 8.

been carried through 30 passages in fertile eggs. Its ID_{50} for mice in 15th, 21st, 24th and 30th egg passages was 10^{-7}, based on titrations of Chamberland L_3 filtrates of 1% suspensions of whole embryo tissues in half-strength Martin's peptone solution, the filtrates themselves showing a titer of 10^{-5}. This titer was reached by the 4th or 5th day. The average incidence of embryo deaths between the 2nd and 6th days was 79% in inoculated eggs incubated at 35°C, and 46% in eggs incubated at 37.5°C. The virus content of the embryo tissue did not appear to be influenced by the temperature at which the inoculated eggs were incubated.

Up to the 18th passage, the material used to inoculate the next group of eggs consisted of the heads and necks only of the embryos from the preceding passage. After the 18th passage, the entire embryo was used in preparing the passage material. Nine-day eggs were employed in making the passages. All were inoculated by the chorioallantoic route. The inoculum in all passages consisted of 0.1 ml of Chamberland L_3 filtrates of 1% suspensions of the chick embryo tissue in half-strength Martin's peptone solution. Twelve supplementary passages were made by the yolk sac route. In the latter, the passage material consisted of filtrates prepared from suspensions of abdominal viscera only. This series showed about the same virus content at the end of the 12th passage as the series carried with material from the heads and necks only. In the series inoculated by the chorioallantoic route, the allantoic fluid, unfiltered, showed an ID_{50} of 10^{-5} in the 9th passage, the chorioallantois itself an ID_{50} of 10^{-4} in the 5th passage, and the yolk only in the 15th passage titered only 10^{-1}.

Embryos from 5 to 14 days of age seemed to serve equally well for the propagation of the virus. However, in eggs containing 15- and 16-day-old embryos the ID_{50} dropped from the usual level of 10^{-7} to 10^{-4}, and in 17-day-old eggs to zero. With this drop in titer the incidence of embryo deaths also fell off. Attempts to adapt the virus to older embryos failed.

Neutralization tests on material from the 24th egg passage did not show any appreciable change in the antigenic properties of the virus.

Murine MM Strain. This strain, described by Jungeblut and Dalldorf[2] was obtained from Dr. Jungeblut in 1946 in its 107th mouse passage. Jungeblut[3] briefly mentions that this strain had been "carried over five serial passages in fertilized hen's eggs." In a later paper, Brutsaert, Jungeblut and Knox[4] state that the virus could not be recovered after the 5th or 6th serial passage.

We succeeded in carrying the MM strain through 10 egg passages under conditions similar to those employed for the SK strain. The ID_{50} for mice of material from the 10th egg passage was about 10^{-6}, based, as in the preceding series, on serial dilutions of Chamberland L_3 filtrates of 1% suspensions of whole embryo tissue. At 35°C the average incidence of embryo deaths was 75%, based on a total of 52 eggs employed; at 37.5°C this incidence dropped to about 20%.

Murine Lansing or C(M) Strain. Of especial interest have been the results obtained with what appears to be a murine high titer variant of the Lansing strain. This strain prior to its trial in eggs had been passed continuously in mice over a period of more than four years. Just when it first showed itself as a high titer virus we are unable to say since actual titrations were not carried out during most of this period. Up to 1943 it behaved like a typical "low titer" Lansing strain. However, shortly before the present studies were undertaken in 1946, it showed an ID_{50} of 10^{-7} for mice. The possibility that the supposed variant was actually a foreign virus obviously had to be considered. An investigation was therefore undertaken to determine its relationships to Lansing "passage strains" from other laboratories. This study is still in progress, and will be reported later.[5] Its inclusion in this report—as a probable variant of the Lansing strain—seems

[2] Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

[3] Jungeblut, C. W., *J. Exp. Med.*, 1945, **81**, 275.

[4] Brutsaert, P., Jungeblut, C. W., and Knox, Alice, *J. Pediat.*, 1946, **29**, 350.

to be justified by the results of serological observations thus far and by the fact that it has produced characteristic flaccid paralysis in individual monkeys when these were inoculated intracranially with mouse brain-cord virus suspensions. In one group of 3 monkeys inoculated with "autolyzed" brain-cord material from recent mouse passage prepared according to Milzer and Byrd,[6] all developed typical paralysis with characteristic cord lesions.

This virus has been carried through 15 egg passages. As in the murine SK series the passages were initiated with a Chamberland L_3 filtrate of a 1% suspension of mouse brain-cord tissue in half-strength Martin's peptone solution. The ID_{50} of this filtrate was 10^{-5}, or not less than 10^{-7} for the unfiltered material. This was seeded to 5 eggs in 0.1 ml amounts, by the chorioallantoic route. All subsequent passages were made as described for the preceding viruses. The virus has been carried through the 15 passages without apparent loss of its ID_{50} for mice. Up to the 5th passage, the material passed consisted of heads and necks only of the preceding embryos. In all subsequent passages filtrates of ground whole embryo tissue were employed. Of a total of 78 eggs inoculated in making these passages, 76% developed dead embryos between the 2nd and 6th days.

While the infectivity for mice of the egg passage material has remained high (10^{-7}), its infectivity for monkeys has proved to be low. An undiluted L_3 filtrate of a 1% suspension of material harvested from the 5th egg passage was inoculated into 4 Rhesus monkeys, each animal by both the intracerebral and intraperitoneal routes. No symptoms were observed until the 3rd week, at which time all 4 of the animals developed a well-defined awkwardness in their movements. None developed a definite paralysis, however. Three of the animals were sacrificed soon after the onset. No well-defined cord lesions were observed in any of them, and only one showed perivascular cuffing in the brain. The 4th animal recovered without any residual manifestations. A similar filtrate from the 10th egg passage was inoculated, undiluted, into 2 monkeys. Both failed to develop symptoms, despite the fact that the ID_{50} of this particular filtrate, for cotton rats, was 10^{-4} and 10^{-5} for mice (or not less than 10^{-6} and 10^{-7} respectively, for the unfiltered material). Employed in a dilution of 10^{-4}, this filtrate was neutralized by an equal volume of anti-C(M) serum produced in a rabbit (final serum dilution 1:5), but not by antisera against the murine SK strain and the GD VII strain of Theiler's virus when these antisera were employed in similar dilutions. Certain tests carried out with mouse brain-cord suspensions have shown, however, that there is some cross reaction with the murine SK strain.[5]

Theiler's Encephalomyelitis Virus. The GD VII strain of Theiler's virus was carried through 10 egg passages. Success with strains of Theiler's virus had been reported, however, by earlier workers.[7,8,9] The ID_{50} of an L_3 filtrate of material from the 10th passage, prepared from a pool of whole embryos, was 10^{-5}. With this particular virus the average incidence of embryo deaths was only 23%, based on a total of 55 eggs employed. The virus was neutralized by an homologous antiserum produced in a rabbit but not by anti-murine SK serum nor by anti C(M) serum.

Strains Which Failed to Grow in Fertile Eggs. The following strains failed to show growth in developing eggs: (1) the cavian SK strain,[10] obtained from Dr. Claus W. Jungeblut in 1946 as his 74th serial passage in guinea pigs, (2) the original monkey passage SK strain,[11] obtained from Dr. John

5 Schultz, E. W., and White, S. C., unpublished observations.

6 Milzer, A., and Byrd, C. L., *Science*, 1947, **105**, 70.

7 Gard, S., *Acta Med. Scand.* (Suppl), 1943, 1943.

8 Dunham, W. B., and Parker, Sue, *J. Bact.*, 1943, **45**, 80.

9 Riordan, J. T., and Sá-Fleitas, M. J., *Science*, 1946, **103**, 449.

10 Jungeblut, C. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 177.

11 Trask, J. D., Vignec, A. J., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 147; 1939, **41**, 241; *J. A. M. A.*, 1938, **111**, 6.

R. Paul in 1946 in its 22nd passage, (3) a cotton rat passage strain of the Lansing virus, obtained from Dr. Hubert Loring in 1946, (4) the MV strain, and (5) the FW strain isolated by one of us (E.W.S.) from the stools of a poliomyelitis patient in 1941. All 5 of these strains failed to prove infectious for test animals after the 5th or 6th egg passages. Since the L_3 filtrate of the Lansing strain had failed to induce infection in mice, the egg passages in this series were initiated with 0.2 ml of an unfiltered 1% suspension mouse brain-cord tissue in half-strength Martin's peptone solution of the passage material, which did prove infectious for 5 out of 6 cotton rats.

After we had completed this work a paper by Riordan and Sá-Fleitas[12] appeared in which they also report having obtained negative results with certain "monkey pathogenic mouse adapted strains" of poliomyelitis virus.

Summary. Further observations on the cultivation of the murine SK strain of poliomyelitis virus in fertile eggs are reported. This strain has now been carried through 30 passages in eggs. The virus has been found to be widely distributed in the infected embryo. It is easily transmitted from egg to egg by different routes. Embryos from 5 to about 14 days of age seem to serve equally well for the propagation of the virus, but those above 16 days of age fail to support its growth.

The murine MM strain was carried through 10 egg passages; a Stanford mouse passage strain, believed to be a high titer variant of the Lansing strain, tentatively labeled C(M) virus, was carried through 15 egg passages. The GD VII strain of Theiler's mouse encephalomyelitis virus was carried through 10 passages. It caused the lowest incidence of embryo deaths of the three viruses cultivated. Five strains of poliomyelitis virus failed to show growth in eggs.

[12] Riordan, J. T., and Sá-Fleitas, M. J., *J. Immunol., Virus Research and Exp. Chemotherapy*, 1947, **56**, 263.

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Ability of Diisopropylfluorophosphonate (D.F.P.) to Produce Antidromic Activity in Motor Nerves.

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Masland and Wigton[1] demonstrated antidromic activity in motor nerves, occurring in association with fascicular twitching of the muscles produced by neostigmin (Prostigmin), or by eserin. They concluded that the cholinesterase inhibiting action of these drugs led to an accumulation of acetylcholine, which stimulated the motor nerve at its ending, and initiated antidromic impulses. This work was further extended by Eccles, Katz, and Kuffler.[2] These investigators demonstrated that the antidromic activity observed in the motor nerve sometimes originated in the muscle fibre, and at other times originated in the nerve ending itself.

In view of the development of new anticholinesterase drugs, it was of interest to determine whether the new drugs produce similar antidromic activity. A study similar to that previously reported with neostigmin has therefore been made using diisopropylfluorophosphonate (D.F.P.). The drug was injected intramuscularly or intraperitoneally in cats, in doses of 5 to 10 mg, as a 1% solution in peanut oil. Active fascicular twitching of the muscles was usually observed in about 40 minutes from the time of injection. When the drug was injected intramuscularly,

[1] Masland, R. L., and Wigton, R. S., *J. Neurophysiol.*, 1940, **3**, 269.

[2] Eccles, J. C., Katz, B., and Kuffler, S. W., *J. Neurophysiol.*, 1942, **5**, 211.

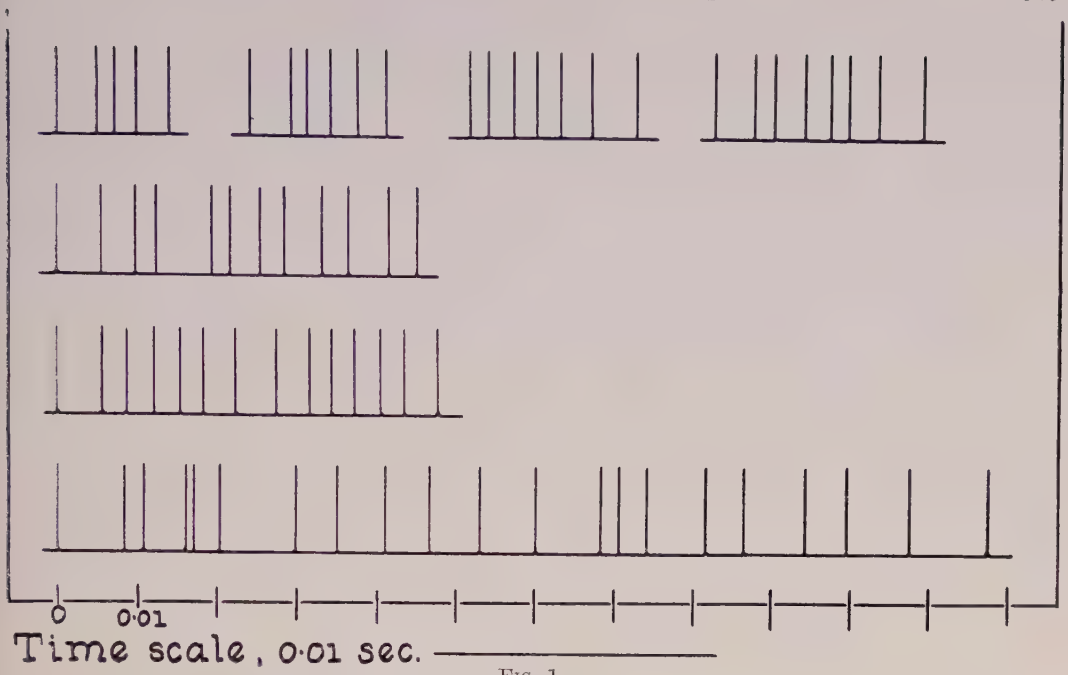

FIG. 1.

Diagram showing the spike frequency and the duration of 7 typical grouped discharges produced by D.F.P. and recorded from an anterior root of the cat. The bursts diagrammed contain from 5 to 21 spikes. Bursts consisting of a single spike, which also occurred, have not been indicated.

the twitching usually appeared first in the limb into which the drug had been injected. When recording electrodes were applied to the motor nerve roots within the spinal canal under these conditions, antidromic activity was recorded. The activity consisted of repeated bursts of nerve impulses, the impulses occurring in groups of 1 to 20, at frequencies up to 200/second during the bursts. The activity appeared to be identical with that previously reported in connection with the administration of neostigmine. (Fig. 1).

These experiments provide further evidence of the similarity of action of eserine, neostigmine, and D.F.P., and support the view that the effect of these drugs on the neuromuscular system is a result of their common action as cholinesterase inhibitors.

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Galactose Excretion in Young and Hepatoma Rats Fed Skim Milk Diets.*

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Adult rats receiving liquid skim milk excrete as high as 35% of the ingested galactose in the urine. When whole milk or skim milk supplemented with 3 or 4% fat is fed, the urinary loss of galactose is greatly reduced.[1] Fat increases the utilization of galactose by

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Evaporated Milk Association, Chicago, and the National Dairy Council, Chicago, in behalf of the American Dairy Association.

[1] Schantz, E. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1938, **122**, 381.

the rat when either lactose or galactose is ingested in milk or synthetic type rations. The percent of the ingested galactose lost in the urine is independent of the actual amount of galactose ingested but is dependent upon the per cent of galactose in the ration.[2,3] The rate of intestinal absorption of galactose varies inversely with the percentage of fat in the diet.[3]

Heretofore, in the galactose excretion studies, adult rats were employed. This report presents results of tests with weanling rats and rats with hepatoma.

Weanling male albino rats of the Sprague-Dawley strain were kept in individual wire mesh metabolism cages. Urine samples were collected under toluene for 24-hour periods. Once or twice weekly the galactose content was determined by using a slight modification of the Shaffer-Hartmann method[4] with a factor of 1.22 to convert the values to galactose. Numerous urine samples were inoculated with *Saccharomyces cerevisiae* to test for fermentable reducing substances and in no case did this cause the amount of reducing substances to decrease.

The diet of the animals was prepared fresh daily by incorporating 10 g of commercial skim milk powder in water to a volume of 100 cc. To each 100 cc were added 12 mg of ferric pyrophosphate (Mallinckrodt N. F. VII), 0.6 mg of $CuSO_4 \cdot 5H_2O$ and 0.6 mg of $MnSO_4 \cdot 4H_2O$. The liquid diet contained 4.65% lactose of 2.45% galactose. The fat soluble vitamins were supplied as a corn oil concentrate, 2 drops per week furnishing 1.568 mg of α-tocopherol, 0.147 mg of 2-Me-1,4-naphthoquinone, 0.35 mg of β-carotene,† and 0.0098 mg of calciferol.‡

Four duplicate experiments, consisting of 6 rats each, were set up at different intervals. The average weekly galactose excretion values obtained with each group are given inTable I

The studies were repeated on rats with hepatomas,§ induced by p-dimethylaminoazobenzene or m'-methyl-p-diaminoazobenzene.[5] The hepatomas were ascertained by palpation. The results of these tests are presented in Table II.

Discussion. The urinary excretion of galactose in the weanling rat was lower than that of the adult animal on the skim milk diet. The excretion values (Table I) increased during the fifth and sixth week until the rate of excretion of the adult rat was reached. During the first few days on the liquid diet slight weight losses were observed in each group of rats, but thereafter slow growth occurred. Diarrhea was surprisingly absent in most of the young animals. When present, the excretion value was omitted due to unavoidable contamination of the urine sample. After 3 or 4 weeks on the experimental diet, 55% of the young rats had developed an opacity of the lens. This incidence was surprisingly high. Krewson *et al.*[6] had reported that the lactose content of skim milk alone was not great enough to produce cataracts. Their work differed from this in that their rats were beyond the weanling stage, weighing 80 to 100 g.

Adult hepatoma rats fed the skim milk diet showed excretions of galactose varying from zero to 1/5 the amount excreted by normal adult rats. A slow increase in excretion was noted in Rats 1 and 5 from Groups 2 and 3 respectively. Autopsy of these 2 animals showed no evidence of hepatoma. The high mortality of the tumor animals tended to obscure the results and prevent the completion of most of the experimental runs.

The lower excretion in the weanling and tumor rats approximates that obtained when 3 or 4% of fat is incorporated in the milk.

2 Geyer, R. P., Boutwell, R. K., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **162**, 251.

3 Nieft, M. L., and Deuel, H. J., Jr., *J. Biol. Chem.*, 1947, **167**, 521.

4 Osgood, E. E., *A Textbook of Laboratory Diagnosis with Clinical Applications for Practitioners and Students*, Philadelphia, 1935, 301.

† 90% β-carotene and 10% α-carotene.

‡ Crystalline irradiated ergosterol.

§ The hepatoma animals were obtained through the courtesy of Dr. C. A. Baumann and coworkers.

5 Rusch, H. P., Baumann, C. A., Miller, J. A., and Kline, B. E., *A. A. A. S. Research Conference on Cancer*, 1944, 267.

6 Krewson, C. F., Schantz, E. J., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 573.

TABLE I.
Galactose Excretion by Male Weanling Rats.

Group	Weekly galactose excretion values* (% of galactose ingested)										No. of animals with lens opacity
	1	2	3	4	5	6	7	8	9	10	
1	5.69	8.66	8.60	9.7	12.02	—	—	21.7	25.1	—	5
2	7.41	6.46	5.03	3.6	12.20	20.5	20.9	27.7	—	—	2
3	—	6.90	4.45	10.3	11.40	20.1	19.1	32.0	40.0	31.0	3
4	4.61	4.61	8.35	11.56	14.40	16.77	19.8	29.1	—	—	3

* Each group consisted of 6 animals, but contamination due to diarrhea sometimes occurred. Because the contaminated samples were omitted, each figure in the table represents the average urinary galactose excretion of 3 to 6 animals.

TABLE II.
Galactose Excretion by Male Rats with Hepatoma, Fed Skim Milk.

Group	Rat	Weekly galactose excretion values* (% of galactose ingested)									
		1	2	3	4	5	6	7	8	9	10
I	1	5.05	5.05	Dead							
	2	2.98	3.38	6.49	Dead						
	3	2.98	Dead								
	4	0.0	4.3	3.38	5.37	4.78	2.39				
	5	—	—	5.97	8.96	9.23	Dead				
	6	—	—	0.0	3.78	4.78	2.98				
II	1	5.97	6.75	12.30	17.6	24.6	26.0				
	2	6.73	2.58	Dead							
	3	5.69	5.15	4.50	Dead						
III	1	5.97	—	—	0.0	—	—	3.78	5.97	Dead	
	2	6.97	—	—	0.0	—	—	5.97	0.0	Dead	
	3	9.25	—	—	2.98	—	—	6.75	Dead		
	4	4.55	—	—	1.79	—	—	4.30	2.98	4.08	5.97
	5	3.38	—	—	1.79	—	—	20.01	25.40	31.00	33.40
IV	1	4.80	9.4	Dead							
	2	2.92	Dead								

* Each figure represents the galactose excretion value of one animal.

In the young animal more efficient utilization of galactose may be necessary for the more adequate utilization of the high lactose diet during early life. Evidence that the system involved may be one present in the liver is substantiated by the results obtained in rats with hepatomas.

Kosterlitz[7] and others have shown that an increased amount of glucose is found in the liver during galactose assimilation. Greenstein[8] reports that rat hepatoma possesses an acid phosphatase activity double that of normal liver and an alkaline phosphatase activity 120 times as great. This may explain the decreased galactose excretion in the tumor animal. A similarly increased phosphatase system may occur in the young animal, and induce a greater conversion of galactose-1-phosphate to glucose or glycogen. In the older, more mature animal the lactose intake is smaller and the galactose utilization system must compete to a lesser degree. The fact that the adult liver can convert galactose at a certain rate forms the present basis for the galactose tolerance test as a measure of liver function. This study would seem to indicate that the test may be inadequate in determining dysfunction due to tumor.

The hepatomas were ascertained to be originally present through palpation. However, in 2 observations autopsy failed to show

[7] Kosterlitz, H. W., *Biochem. J.*, 1943, **37**, 181.

[8] Greenstein, J. P., *J. Nat. Cancer Inst.*, 1942, **2**, 511.

any evidence of liver tumor. Whether coincidental or not, the influence of the liquid skim milk diet on the resolving of hepatomas and other related disturbances is worthy of investigation.

Conclusions. The urinary excretion of galactose in the young rat is lower than that of the adult animal when a supplemented skim milk diet is fed.

On such a diet, adult rats, with hepatoma induced by azo dyes, excrete a lower percentage of galactose in the urine than do normal adult rats.

16153

A Method for Producing Sustained High Penicillin Levels in the Blood.

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(Introduced by Gregory Shwartzman.)

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Improvement in antibiotic therapy may be expected if high penicillin levels could be maintained in the blood stream and tissues for prolonged periods. The required period of therapy may perhaps thereby be shortened and infections due to exceptionally resistant organisms may more readily be brought under control. Furthermore, high, sustained penicillin blood levels may result in tissue saturation and greater penetration of the drug into foci of infection.[1]

To date, efforts to achieve prolonged levels consisted in the employment of two methods, (a) the use of oil, wax and other menstrua to delay absorption of penicillin introduced into a local depot and (b) the use of drugs capable of delaying the ordinarily rapid excretion of penicillin through the kidneys, *i.e.* para-aminohippuric acid,[2] and caronamide.[3] Caronamide (4′-carboxyphenylmethanesulphonanilide) has been shown by Beyer[4] to have an inhibitory effect on the renal tubular excretion of penicillin. In addition attempts have been made to obtain high levels by continuous intravenous drip and by the periodic administration of large "booster" doses of the antibiotic. The dose and speed of injection have been limited under the continuous intravenous method because of thrombo-phlebitis.

In this study, we have achieved sustained and unusually high levels of penicillin in the blood stream by the periodic intravenous injection of large doses of crystalline penicillin* when caronamide† was used as an adjuvant to block rapid excretion through the kidney.

Initially it was planned to obtain high intracardiac levels of penicillin by introducing the drug through an intracardiac catheter passed into the right ventricle or pulmonary artery. Preliminary experiments on dogs demonstrated, however, that the levels‡ in the aorta achieved by rapid injection of equally large doses of penicillin in concen-

1 Gerber, I. E., Shwartzman, G., and Baehr, G., *J. A. M. A.*, 1946, **130**, 761.

2 Beyer, K. H., Flippin, H. F., Verwey, W. F., and Woodward, R., *J. A. M. A.*, 1944, **126**, 1007.

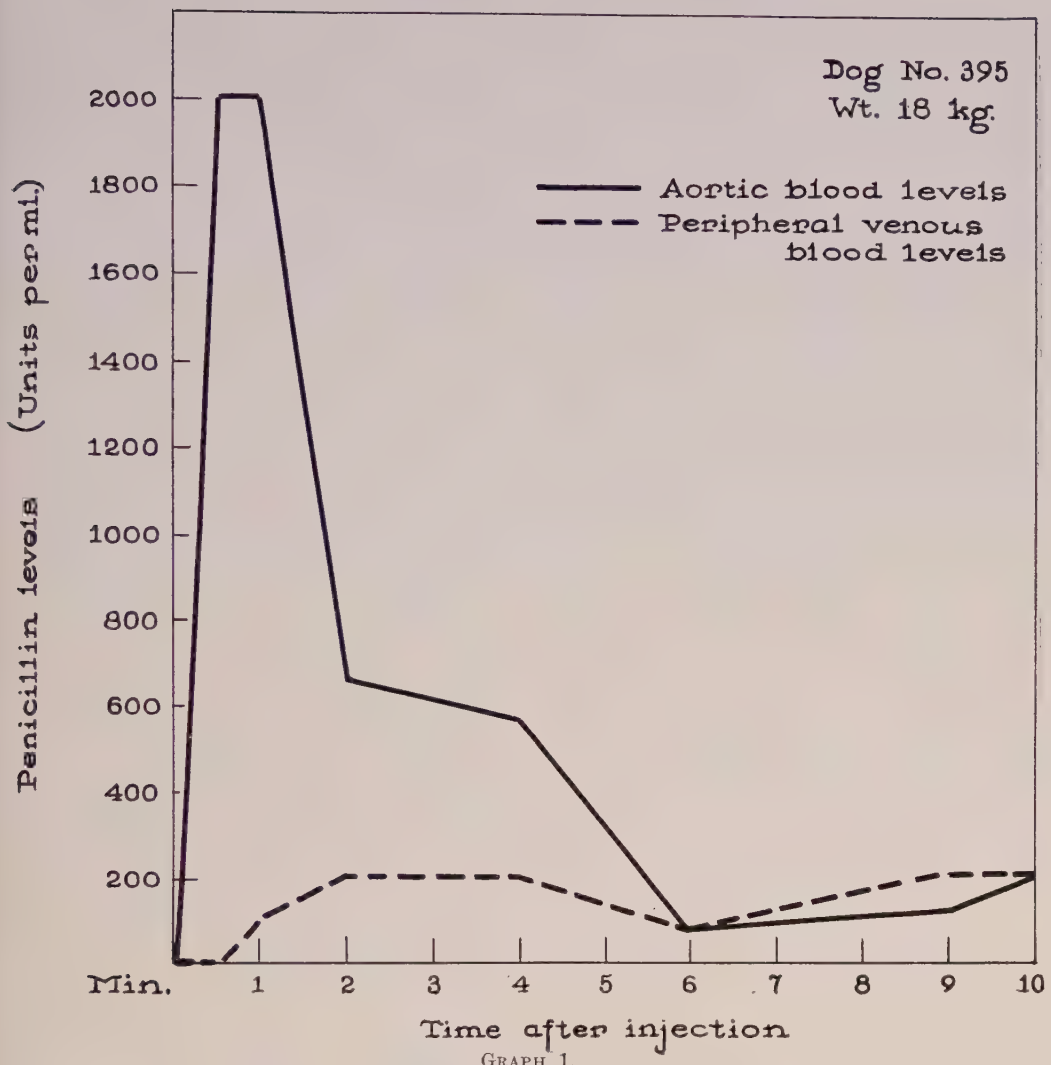
3 Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, A. K., *J. A. M. A.*, 1947, **134**, 1528.

4 Beyer, K. H., *Science*, 1947, **105**, 94.

* Sodium and calcium salts of crystalline penicillin kindly supplied by Schenley Laboratories, Inc.

† Kindly supplied by Sharp and Dohme, Inc.

‡ The method of penicillin assay employed is a broth tube dilution method using *Staphylococcus aureus* H as the test organism and fresh meat extract broth as the medium. The minimal concentration of the standard penicillin required to inhibit the inoculum of 5 x 10^2 *Staphylococcus aureus* H cells was 0.02 units per ml. The standard penicillin was obtained from the U. S. Department of Agriculture. All titrations of serum levels were accompanied by and compared with this standard.



GRAPH 1.

500,000 units crystalline penicillin dissolved in 5 cc distilled water injected rapidly into right brachial vein through an 18-gauge needle.

trated solution into the brachial vein were as great as those that could be obtained by introducing the penicillin directly into the right side of the heart. In the animals, in addition to extremely high levels in the aorta immediately following injection there were found unusually elevated peripheral blood levels. No untoward manifestations supervened when one million units of crystalline penicillin, dissolved in 5 cc of distilled water, were injected as rapidly as possible through a large (18 gauge) needle into the brachial vein of a dog weighing 18 kg (55,600 units/kg). A continuous electrocardiogram taken before, during and following the injection failed to show any alterations in rate, rhythm, or contour of the complexes.

Following these preliminary experiments, the method was applied to patients. The first patient received one million and the second, 2 million units of crystalline penicillin, dissolved in 5 cc of distilled water. The solutions were injected rapidly into the right antecubital vein through a 15-gauge needle, the injection being completed within 5 seconds. No clinical or electrocardiographic abnormalities were noted.

The levels of penicillin in the aortic blood

TABLE I.
Effect of Caronamide upon Penicillin Blood Levels in Units per ml in Patients Receiving a Single Large Dose of Crystalline Penicillin (One Million Units).

	Rapid Intravenous Injection								Intramuscular inj.	
	Patient 1		Patient 2		Patient 3		Patient 4			
Time after inj.	No caronamide	With caronamide	No caronamide	With caronamide	No caronamide	With caronamide	No caronamide	With caronamide	No caronamide	With caronamide
1 min.	112	152	88	152	136	160	—	—	—	—
3 "	132	152	112	152	152	160	—	—	—	—
5 "	120	136	112	136	128	144	32	32	32	32
10 "	104	88	—	112	88	136	32	40	32	40
15 "	64	72	40	72	56	96	40	—	—	—
20 "	—	—	—	—	—	—	40	48	—	48
30 "	40	56	32	48	32	80	32	48	32	48
1 hr	16	48	16	40	8	48	24	48	24	48
2 "	5.7	13	6.6	20	4.4	20	10	20	10	20
3 "	2	10	2	13	0.8	13	4.4	13	4.4	13

obtained from the descending aortic arch during operation, in a human subject weighing 58 kg were determined. At 1, 3, 5, and 10 minutes following rapid injection into the left antecubital vein of one million units of crystalline penicillin (17,300 units per kg) dissolved in 5 cc of distilled water the levels were found to be 480.0, 346.0, 320.0 and 106.6 units per ml respectively.

The peripheral venous blood levels were then studied in human subjects under the following conditions: one million units of crystalline penicillin dissolved in 5 cc of distilled water were injected rapidly into 3 patients prior to and, again later, following the administration of caronamide. For comparison the same dose was given intramuscularly in a control patient, also preceding and following caronamide administration. The results are given in Table I. In all patients receiving caronamide the dose employed was 4 g orally every 3 hours day and night starting 24 hours before antibiotic therapy and continuing throughout the period of observation.

As may be seen from Table I, a very high blood level still persisted one hour after each injection, especially when the patient was receiving caronamide. The peak level in the venous blood from the opposite arm was reached within the first 3 minutes and was equally high in patients under caronamide therapy and without it. There was a progressive fall during the 3 hours of observation, the most precipitous drop occurring during the first 30 minutes. At the 3-hour period the level in the patients receiving caronamide was 5 to 15 times higher than in the same patients without caronamide.

It was thought that the high level still present at the end of the first hour could be maintained or exceeded if the intravenous injection of the same dose was repeated hourly. Table II shows the levels achieved in 3 patients receiving rapid intravenous injections of one million units of penicillin hourly for 10 hours. Two of these patients received caronamide and one did not. Table II also shows the blood levels found after administration of the same total dose (10 million units in 10 hours) by slow intravenous drip in a

TABLE II.

Peripheral Blood Penicillin Levels in Units per ml After the Administration of 10 Million Units of Crystalline Penicillin in 10 Hours.

	One million units of crystalline penicillin, dissolved in 5 cc distilled water, rapidly inj. into right antecubital vein, hourly, for 10 hrs			Ten million units of crystalline penicillin, dissolved in one L. physiological saline, administered by slow intravenous drip in 10 hrs		
Time	Patient 5 Caronamide	6 Caronamide	7 No caronamide	Time, hr	8 Caronamide	9 No caronamide
After 1st injection:						
3 min.	184	184	120	1	27	20
5 "	160	120	80	2	96	32
30 "	96	88	24	3	96	48
55 "	64	64	8	4	104	40
				5	112	48
After 5th inj.:				6	120	40
3 min.	224	228	128	7	136	32
5 "	208	176	96	8	128	—
30 "	104	144	24	9	128	48
55 "	72	96	—	10	136	56
				10½	128	
After 10th inj.:						
3 min.	280	256	120			
5 "	280	240	64			
30 "	232	240	56			
55 "	208	200	16			
24 hr after start	13	5.7	0.5	24 hr after start	8	<.05

patient treated with caronamide and in one, in the absence of the drug.

No toxic effects were noted in any of the cases studied. Bleeding and coagulation times and clot retraction remained normal.

It is apparent from these experiments that higher peak levels are obtained and that higher interim levels are maintained by the hourly rapid injection of a concentrated solution of crystalline penicillin than by giving the same amount of the antibiotic during the same period by means of the usual slow continuous method of intravenous administration. In addition, a summation effect is indicated by the levels recorded after the first, fifth and tenth injections in patients No. 5 and 6 who received caronamide. This indicates that the repeated hourly intravenous injection of such large amounts either still further reduced the renal excretion rate or resulted in tissue saturation so that the fall of blood level by movement of the penicillin into the tissues was reduced.

The high levels of penicillin in the peripheral blood, just described, are the result of the hourly and rapid intravenous administration of extremely high dosage in combination with inhibition of renal excretion by caronamide. The intraaortic peak levels are many times higher than those in the peripheral blood when the antibiotic is injected rapidly in concentrated solution. It is believed that even higher sustained levels could be safely achieved by larger single doses.

Studies are now in progress to determine the effect of such high sustained levels in the peripheral blood and high aortic levels of penicillin upon the rapid sterilization of the vegetations in bacterial endocarditis and upon other acute and chronic local and general infections caused by refractory microorganisms. It is also important to determine the effectiveness of this method in shortening the duration of therapy in these diseases and, perhaps, reducing the total amount of penicillin required.

Summary. A method for obtaining high sustained blood levels of penicillin is described. This consists in repeated large intravenous doses of crystalline penicillin, injected rapidly, in patients receiving caronamide. Clinical applications are suggested.

We wish to thank Miss Beatrice Toharsky for capable technical assistance.

16154

Effect of Acetylsalicylic Acid Ingestion on Electrophoretic Patterns of Human Plasma.*

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In their work with the anti-clotting agent dicoumarol, Link *et al.*[1] presented *in vitro* evidence that salicylic acid was a degradation product of the widely used antipyretic and analgesic acetylsalicylic acid. Following the work of Link, Field[2] discovered that lactating rats, which normally have high levels of fibrinogen and prothrombin, suffered from hypoprothrombinemia after ingestion of dicoumarol; if dicoumarol was replaced by acetylsalicylic acid a much less severe hypoprothrombinemia resulted. Rapoport and Guest[3] found a decreased sedimentation rate of erythrocytes in subjects receiving salicylic acid or its acetyl derivative, but admitted that their results may have been influenced by certain uncontrolled factors. They contended that the chief factor in regulation of sedimentation rates was plasma fibrinogen. This statement was confirmed by Allen *et al.*;[4] however, these investigators found no hepatic pathology in dogs fed fatal doses of dicoumarol. It was claimed by Homburger[5] that salicylates produce a fall of plasma fibrinogen proportional to total dose rather than to blood levels. His results are subject to question since they are based on a study of only 3 patients, and these had metastatic carcinomas. It has also been claimed by Shapiro, Redish, and Campbell[6] that the ingestion of acetylsalicylic acid increases the clotting time of the blood.

All of the work cited above was based either on the method of Quick[7] or of Cullen and Van Slyke.[8] There are definite theoretical disadvantages in both of these. Quick's method measures clotting time, and depends on the addition of calcium and thromboplastin to a diluted plasma. The dilution is unphysiological and is also subject to salt errors.[9] In Cullen's method a fibrin precipitate is weighed and analyzed for nitrogen; the results are expressed as either prothrombin or fibrinogen. Since the stoichiometry of fibrin formation *in situ* is not known, the method is at best somewhat equivocal.

It appeared to us that electrophoresis might be applied advantageously to this problem, since it is capable of separating the plasma proteins with a minimum of denaturation and loss. While prothrombin is not ordinarily present in sufficient concentration to appear on an electrophoretic pattern, fibrinogen is a well defined component of such patterns.

Materials and Methods. Samples of oxalated blood were obtained from normal healthy male and female subjects before and after a 7-day period during which they ingested 4 g of acetylsalicylic acid per day. The plasma was diluted with 3 volumes of a barbiturate buffer (pH = 8.63, ionic strength—0.1, sodium diethyl barbiturate—0.1N), and dialyzed at 2°-6°C for 3 days with daily change of buffer. Dialysis was performed in Visking casings. The final volume of buffer

* Aided by a grant from the Institute for Study of Sedative and Analgesic Drugs and Bristol Laboratories.

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2 Field, J. B., *Am. J. Physiol.*, 1945, **143**, 238.

3 Rapoport, S., and Guest, G. M., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 43.

4 Allen, E. V., Barker, N. W., and Waugh, J. M., *J. Am. Med. Soc.*, 1942, **120**, 1009.

5 Homburger, F., *Am. J. Med. Sciences*, 1946, **211**, 346.

6 Shapiro, S., Redish, M. H., and Campbell, H. A., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 12.

7 Quick, A. J., *J. Am. Med. Soc.*, 1938, **110**, 1658.

8 Cullen, G. E., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, **41**, 587.

9 Deutsch, H. F., and Gerarde, H. W., *J. Biol. Chem.*, 1946, **166**, 381.

TABLE I.
Electrophoretic Analysis of Plasma Proteins.

Case No.	Normals						Experimentals					
	Alb.	Globulins					Alb.	Globulins				
	A	α_1	α_2	β	Φ	γ	A	α_1	α_2	β	Φ	γ
1	58.5	5.71	7.38	10.71	5.98	11.83	57.3	6.42	7.74	11.84	5.76	11.00
2	56.6	5.80	9.20	11.10	5.50	11.90	57.5	4.10	9.47	13.38	6.31	9.28
3	57.2	2.96	9.98	11.60	8.90	9.70	53.5	3.00	10.70	14.40	8.75	9.70
4	66.8	4.46	8.00	11.24	3.42	6.09	66.9	6.37	7.33	10.85	3.82	4.77
5	57.8	5.41	10.80	11.94	5.40	8.80	57.8	4.53	11.83	14.04	4.76	7.48
6	67.8	4.24	8.47	9.54	3.18	6.71	64.2	6.60	8.80	9.74	4.07	6.61
7	55.9	4.86	10.77	12.15	6.25	10.08	56.0	5.73	7.29	12.50	6.25	11.97
8	64.6	4.98	6.64	11.00	4.67	8.15	61.0	6.67	9.60	11.92	4.36	6.38
9	61.1	5.85	11.99	8.77	6.43	5.85	62.7	4.94	10.64	7.99	5.70	7.98
10	61.1	3.97	10.11	11.55	4.33	9.04	63.7	4.24	8.50	11.89	3.68	7.92
11	61.7	3.40	7.71	9.25	5.24	12.67	61.4	4.78	7.97	9.56	4.78	11.56
12	58.7	3.30	10.19	12.28	5.09	10.48	55.9	4.54	9.41	11.37	7.79	11.04
13	68.3	5.40	7.04	11.22	3.85	4.16	66.6	7.47	7.78	9.98	3.74	4.36
14	58.3	4.39	9.86	11.23	7.12	9.04	56.0	5.06	9.11	12.16	7.60	10.13
15	67.7	5.32	9.88	9.51	2.66	4.94	67.7	4.30	9.41	8.06	2.42	8.05
16	62.3	5.30	13.26	9.47	5.69	6.06	61.2	6.08	11.02	11.79	5.30	4.56
Avg	61.5	4.70	9.45	10.78	5.23	8.46	60.6	5.30	9.13	11.34	5.32	8.29
Std. Dev.	4.17	0.90	1.70	1.08	1.52	2.51	4.31	1.07	1.32	1.90	1.68	2.39

Std. Dev. of Changes*	A	α_1	α_2	β	Φ	γ
	1.45	0.83	1.26	0.97	0.76	1.09

* Calculated for each component based on difference between normal and experimental values.

was sufficient to fill the apparatus completely. Electrophoresis was carried out in the Longsworth[10] modification of the Tiselius[11] apparatus. The patterns were photographed and projections (2.5X) were traced on bond paper. The area enclosed by the base line and the protein peaks was measured with a planimeter and equated to total protein. Each peak was measured separately to determine the individual proteins. Mobility measurements were made from the boundary anomaly of the pattern. While both ascending and descending boundaries were compared and analyzed, only data from the descending boundaries is included.[10] Changes in total protein content were checked refractometrically, and standardized against a sample of crystalline serum albumin dissolved in the buffer. No set of samples (normal and experimental) showed a greater than 0.5% variation in protein content by this method.

Conditions of the electrophoretic experiments were carefully controlled. The current was held at a value of 25 ± 0.5 milliamperes, and was applied for 120 ± 1 minutes. The maximum variation of refractive increment was ± 0.0003. The conductivity of each solution was also measured, and the greatest difference observed in any pair of samples was ± 0.0004 ohms^{-1}. In measurements of area by the planimeter, the sum of the areas or the peaks due to protein was 100 ± 0.1%. This agreed with the measurement of the total area within the same limits.

Results. The detailed analysis of each experiment is presented in Table I. Average values agree well with those of other workers,[12,13] and the standard deviations are also in fairly good agreement. Differences between our results and those of others is largely due to the fact that we used the Pedersen[14] method of area division while others have used the method of Tiselius and Kabat.[15] The

10 Longsworth, L. G., *Chem. Rev.*, 1942, **30**, 323.

11 Tiselius, A., *Trans. Faraday Soc.*, 1937, **33**, 524.

12 Dole, V. P., *J. Clin. Invest.*, 1944, **28**, 708.

13 Bieler, M. M., Ecker, E. E., and Spies, T. D., *J. Lab. Clin. Med.*, 1947, **32**, 132.

14 Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge*, p. 296, Oxford University Press, London, 1940.

15 Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, **69**, 119.

latter of these methods introduces errors discussed by Svensson.[16]

Discussion. Our results indicate that for the 16 cases studied, none of the protein components accessible to electrophoresis shows any marked change. Particularly, only 4 of the 16 cases showed a loss of 0.5% in fibrinogen, while as many cases showed a gain of the same magnitude. Witts[17] has published the statement that fibrinogen levels may be reduced 30% before prothrombin time is significantly prolonged. With respect to the earlier claims cited above, our results indicate that the change in clotting time and sedimentation rates following acetylsalicylate ingestion is not due to marked changes in fibrinogen levels. Actually, the presence of any large asymmetric molecule can change sedimentation rate regardless of protein concentration.[18,19] Whether this is due to an effect on protein surface charge or to chemical interaction is not known. Smith[20] claims that salicylates are bound to some plasma proteins.

Ham and Curtis[21] have reported a very careful study of the relation between hepatic function and plasma proteins. These authors concluded that plasma fibrinogen was an especially sensitive index of liver disease or dysfunction. They stated that in damaged livers the plasma fibrinogen was sharply decreased. If these reports are correct then our results indicate that salicylates are not hepatotoxic, and likewise, any effect of salicylates on prothrombin time is not reflected by corresponding changes in fibrinogen levels.

Summary. 1. Blood of normal subjects was examined by electrophoresis before and after ingestion of 4 g of acetylsalicylic acid per day for 7 days. Physical and chemical constants obtained were in good agreement with published values.

2. No marked changes were observed in any protein components accessible to electrophoresis. The conclusion is drawn that the effect of salicylates on blood clotting time and sedimentation rates of erythrocytes cannot be due to a reduction of plasma fibrinogen, and that salicylates are probably not hepatotoxic.

16 Svensson, H., *Ark. Min. Kemi och Geol.*, 1946, **22**A, 1.

17 Witts, L. J., *J. Path and Bact.*, 1942, **54**, 516.

18 Zozaya, J., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 182.

19 Yardumian, K., *Am. J. Clin. Path.*, 1937, **7**, 105.

20 Smith, P. K., Gleason, H. L., Stoll, C. G., and Ogorzalek, S., *J. Pharm. and Exp. Therap.*, 1946, **87**, 237.

21 Ham, T. H., and Curtis, F. C., *Medicine*, 1938, **17**, 413, 447.

16155

Development of Renal Function in Fetal and Neo-Natal Rabbits Using Phenolsulfonphthalein.*

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At birth mammals undergo tremendous physiological adjustments in their respiration, nutrition, circulation and excretion correlated with the achievement of independence of the material circulation. Much attention has been directed toward the first 3 of the above mentioned adjustments but fewer physiological studies have been made on the development of an independent renal excretion. Most of the work reported indicates that there is some formation of urine in the terminal stages of uterine life.[1,2,3] It has been the purpose of our investigations to try to determine to

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what extent there is renal function in fetal rabbits and to attempt to measure the change in the renal excretion at birth and during the first few days of postnatal life. The rabbit was selected as our experimental animal because of the convenience of its breeding habits, its short gestation period and the fact that, like man, it has only a vestigial allantois.[4]

Methods. Phenolsulfonphthalein was selected as our indicator of renal function because it is rapidly excreted by tubular secretion as well as glomerular filtration.[3] It has been widely used since 1910 as a clinical test of renal function in man and is relatively non-toxic.[2] Finally the method of quantitative estimation of this dye in body fluids is simple and accurate.

A number of does were bred, each by a single copulation, in order that young of known age could be obtained. Some of these does were anesthetized before term and the fetuses exposed while others were allowed to deliver in order that newborn of various ages could be obtained. In this manner a series of fetuses and newborn of known age covering the period from the 26th to the 42nd day after conception was obtained. Deliveries occurred between the 30th and the 33rd days after conception.

A subcutaneous injection of one mg of PSP in a volume of 0.5 ml was adopted as the test dose. The percentage of this dose excreted into the bladder in one hour was determined.

In the fetal series 4 mothers were lightly anesthetized with 1.3 g of urethane per kilogram of body weight augmented with ether during the surgery. The uteri were exposed and a small opening made in each horn. Through these openings the urogenital papillae of 2 fetuses were tied off and the PSP injections made subcutaneously with special care to avoid leakage. Then the uterine incisions were sutured and the abdomen closed during the collection period. At the end of the hour the fetuses were removed, sacrificed and the bladder contents washed out for analysis. The survival of the fetuses as indicated by pulsation of the umbilical artery was taken to be a proof of the continued normal function of the placenta.

In the newborn series there were 11 litters. Tests of the rate of excretion of PSP were made at daily intervals after birth. Each young rabbit was injected subcutaneously with the standard dose of PSP and suspended over a beaker. At the end of an hour each was sacrificed, the bladder contents added to any urine voided during the hour and analysed for PSP.

Nine experiments were carried out to determine the placental transmission of PSP from maternal to fetal blood and vice versa. In one series the mother was injected intravenously with 6 mg of PSP in one ml and the fetal blood tested after an interval of one hour for the presence of the dye. In the other series 2 fetuses were injected subcutaneously, each with 3 mg of PSP in 0.5 ml volume. The mother was catheterized and urine collected for a period of one hour during which several maternal blood samples were taken. The maternal and fetal blood samples were obtained by heart puncture.

The concentration of PSP in the centrifuged urine was determined with the aid of a photoelectric colorimeter on the properly diluted urine alkalinized with sodium carbonate. The plasmas were first precipitated with one part of saturated trichloracetic acid to 8 parts of blood, the supernatant portion alkalinized and the color estimated. This method measures only the fraction of the phenol red which is not bound to proteins but avoids the errors accompanying hemolysis.

To test the influence of the anesthetic on the renal excretion of the dye, some of the newborn were given 1.3 g of urethane per kg and the rate of their excretion of PSP simultaneously compared with that of an unanesthetized litter mate.

1 Barclay, A. E., Franklin, K. J., and Pritchard, M. M. L., *The Fetal Circulation,* Charles C. Thomas, Springfield, Ill., 1945.

2 Rowntree, L. G., and Geraghty, J. T., *J. Pharm. and Exp. Therap.,* 1910, **1**, 579.

3 Smith, H. W., *The Physiology of the Kidney,* Oxford University Press, New York, 1937.

4 Windle, W. F., *Physiology of the Fetus,* W. B. Saunders Co., Philadelphia, 1940.

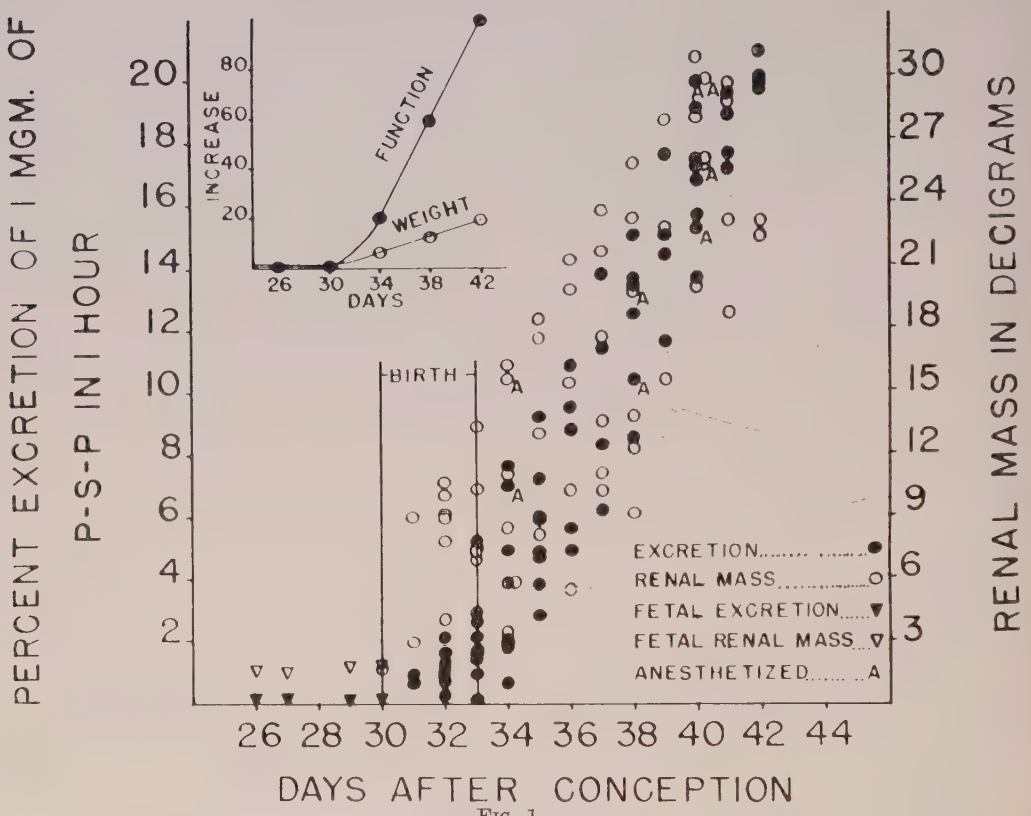

FIG. 1.

The rate of excretion of phenolsulfonphthalein and the combined weights of the 2 kidneys in fetal and newborn rabbits of varying age. The rate of excretion (solid dots) is plotted as the percentage of a 1 mg subcutaneous dose excreted in one hour. The kidney weights (circles) are plotted in decigrams against the ordinate on the right. The absolute rate increase of excretion and renal mass is plotted in the small graph at the upper left, using earliest noted fetal excretion and renal mass values as bases.

The kidneys of fetuses and newborn were removed and weighed after the excretion rate had been determined.

Results. The excretion of PSP by the fetuses in one hour was practically negligible in spite of high plasma concentrations of the dye and showed very little change between the 26th and the 30th day of gestation. (Fig. 1) The weight of the fetal kidneys also showed little change during this period.

Following birth there was a regular increase in the rate of excretion of PSP and in the kidney weight with increasing age. The increase in function was about 5 times greater than the increase in renal mass, *i.e.* the rate of dye excretion at the end of 10 days postnatal life was 100 times greater than the prenatal rate while the renal mass was increased less than 20 fold.

In the experiments comparing litter mates, one of which was anesthetized, there was virtually no effect of the anesthesia on the rate of dye excretion. (Fig. 1)

There was no appreciable transmission of the dye across the placental barrier in either direction during one hour.

Discussion. It is well established that some renal excretion occurs in mammalian fetuses as they approach term.[4,5,6] Gersh has defined qualitatively the beginning of renal excretion in rabbit and other mammalian fetuses by histochemical technique.[7] He found evidence

[5] Daly, Harriet, Wells, L. J., and Evans, Gerald, PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 78.

[6] Smith, C. A., *The Physiology of the Newborn Infant,* Charles C. Thomas, Springfield, Ill., 1945.

of glomerular filtration and tubular secretion even in the mesonephros, but his work does not indicate the relative quantity of materials excreted in the urine. That this avenue of excretion is not important so long as the placenta functions normally is indicated by the observation that fetuses can survive to term without kidneys or with obstructed urinary passages.[4,6,8]

In considering the cause of the rather abrupt change in renal function which we have observed at the time of birth, one must consider both physiological and morphological factors. Hamilton, Woodbury and Woods[9] have measured the blood pressures of fetal and newborn rabbits. They find some increase in the systemic arterial pressure at the time of birth and a gradual continuing increase to the adult level. This seems to be true of other mammalian fetuses as well.[1,4,6] †

On the morphological side there are reports that the glomeruli undergo changes at the time of birth which render them more capable of filtration.[6,10] Gruenwald and Popper suggest that the fully developed visceral layer of Bowman's capsule impairs filtration in the embryonic kidney.[10] They believe that a rupture of this epithelial layer occurs at birth with an expansion of the capillary loops of the glomerulus.

The combined effect of the increase in blood pressure and the increase in filtering surface may cause the sudden increase in renal function observed at birth. The dye then excreted would represent the sum of that filtered at the glomeruli and that secreted by the tubule cells, the dye secreted by the tubule cells being flushed out by the fluid filtered at the glomeruli.

Our failure to find any appreciable placental transmission of PSP in one hour is at variance with the finding of Lell and Liber.[11] These investigators found that 15 to 30% of a dose of PSP injected into rabbit fetuses could be recovered from the urine of the mother in a 6-hour period. This discrepancy with our results may be due to the difference in the time interval if it is not due to differences in the technique of injecting the fetuses.

Summary. 1. Renal function in the rabbit as measured by phenolsulfonphthalein excretion increases rapidly and regularly in the first 10 days after birth but does not exist to an important degree before birth. 2. At the end of 10 days of postnatal life the rate of dye excretion is 100 times greater than the prenatal rate while the renal mass is increased less than 20 fold. 3. In one hour PSP does not traverse the rabbit placenta in either direction in appreciable amounts after the 26th day of gestation. 4. The effect of urethane anesthesia on the excretion of PSP by newborn rabbits was found to be negligible.

7 Gersh, I., *Contr. Emb.*, 1937, **26**, 33.

8 Potter, E. L., *J. Pediat.*, 1946, **29**, 68.

9 Hamilton, W. F., Woodbury, R. A., and Woods, E. B., *Am. J. Physiol.*, 1937, **119**, 206.

† Another possibility is suggested by the work of Truetta *et al.* (*Lancet*, 1946, **251**, 237) who have described a physiological mechanism for diverting the renal blood flow from its normal course in such a way as to render the renal cortex ischemic.

10 Gruenwald, Peter, and Popper, Hans, *J. Urol.*, 1940, **43**, 452.

11 Lell, Wm. A., and Liber, K. E., *Anat. Rec.*, 1928, **38**, 53.

16156

Some Pharmacological Characteristics of Bacitracin
III. Chronic Toxicity Studies of Commercial Bacitracin in the Dog and Monkey.*

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In earlier studies of the toxicity of bacitracin,† nephrotoxic changes were oberved in the mouse, but not in the rat.[1] In further studies of the absorption, distribution, and excretion of bacitracin in the dog,[2] no apparent clinical signs of renal toxicity were observed. Because of these differences, it appeared desirable to study the toxicity of bacitracin further. The effects of prolonged daily administration of the antibiotic to dogs and monkeys were, therefore, investigated.

Comparison of a number of samples of commercial bacitracin clearly indicated that the toxicity varied independently of the activity[1] and since the toxicity was not altered by destruction of the active principle, it seems reasonable to assume that further purification of bacitracin will lead to a lowered toxicity. Sample No. B-100 was much less toxic than the others and was therefore used for the most part in the tests described below.

Experiments in Dogs. Three mongrel dogs, which had been given bacitracin previously in the course of absorption and excretion studies,[2] each weighing between 7 and 9 kg were maintained on a stock diet of Friskies‡ dog meal and water *ad libitum* for a period of 28 days. During this period, control blood counts were performed on each dog at approximately weekly intervals. The data obtained were within normal limits. During this control period, samples of urine were negative for sugar and albumin, and microscopic examination of the sediment was negative. After this period of observation, 1000 units[3] per kg body weight of bacitracin, lot No. B-100, were administered intramuscularly 3 times daily for 5 days of each week. On Saturdays and Sundays 2 doses of 1500 units per kg each were given. The antibiotic, in 2 to 3 cc of water, was injected intramuscularly using a different limb for each injection. The animals received treatment for 24 days. During this period, blood counts were performed at approximately weekly intervals. The red cell count showed no significant variations from the normal. One dog exhibited a moderate, persistent leukocytosis (15,000 per mm^3) with an increase in the polymorphonuclear leukocytes (average, 87%). The other dogs showed normal total leukocyte counts with an increase in the polymorphonuclear leukocytes (85%). Urinalyses disclosed no significant abnormalities. Blood sugar (80 to 95 mg %) and non-protein nitrogen analyses (30 to 48 mg %) after 10 and 23 days of dosing, disclosed no significant changes except in one dog in which the non-protein nitrogen ranged from 55 to 66 mg %. Without control observations, this cannot be unequivocally attributed to the influence of the antibiotic. There were no appreciable changes in body weight. On the last day of the

* The work described in this paper was done under a contract between the Office of the Surgeon General and Columbia University. Administration of the contract was directed by Dr. Frank L. Meleney.

† Bacitracin is the antibiotic discovered by Johnson and associates.[3] The material used in this study was kindly furnished by Dr. John T. Goorley of the Ben Venue Laboratories, Bedford, Ohio.

1 Scudi, J. V., and Antopol, W., PROC. SOC. EXP. BIOL. AND MED., 1945, **64**, 503.

2 Scudi, J. V., Clift, M. E., and Krueger, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 9.

‡ Carnation Company, 450 Seventh Avenue, New York, N.Y.

3 Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, **102**, 376.

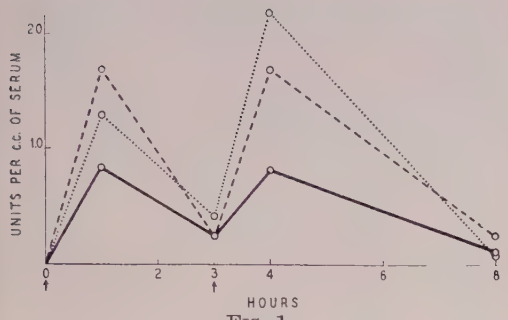

FIG. 1.

Serum concentrations following intramuscular injections of 1000 u/kg in each of 3 dogs. Bacitracin was injected at 0, 3, and 8 hours.

experiment, blood samples taken from each dog were analyzed for their bacitracin content. The data, shown in Fig. 1, indicate that measurable concentrations of the drug following the 3 intramuscular injections persisted in the circulating blood for approximately 13 hours of that day, leaving the blood free of circulating bacitracin for about 11 hours out of the 24.

At the end of the experiment all animals were sacrificed. Macroscopic examination of organs and tissues revealed no significant changes other than local induration at the sites of repeated intramuscular injection.

Microscopic examination of the tissues revealed the following: In the kidney, the cells of the proximal and distal convoluted tubules showed eosinophilia of the cytoplasm. Occasional basophilic globoid bodies were found in the convoluted tubules and a moderate number in the loops of Henle and collecting tubules. An occasional collection of round and plasma cells was found in the stroma. In one dog (No. 156) there was a coagulum in the loops of Henle and the collecting tubules.

The liver appeared to contain considerable glycogen. There was no evidence of cellular damage. The bone marrow was hyperplastic. The spleen showed large follicles and the pulp contained numerous macrophages with brown pigment and megakaryocytes. No significant changes were found. The thyroid appeared hyperplastic. The colloid was vacuolated and in places basophilic. Small nests of epithelial cells were found in dog No. 156. The muscle at the site of injection showed edema and numerous polymorphonuclear leukocytes which extended between the muscle fibres.

Experiments in Monkeys. After a control period of one to 3 weeks, during which time routine blood counts and urinalyses were performed, 5 monkeys were given bacitracin. Monkeys No. 1 and No. 44, each weighing 3 kg, were given 1500 units of bacitracin, lot No. B-100, per kg of body weight morning and evening for 5 days of each week. On weekends the total dose of 3000 units per kg was given at one time. All injections were given into the posterior muscle group of the right thigh, thus concentrating any local effect there might be. In order to compare the effect of 2 different lots of bacitracin, monkeys No. 2, No. 30, and No. 46 were similarly dosed with bacitracin lot No. B-102. The animals in both groups were dosed for 37 and 39 consecutive days, respectively.

During the period of treatment, blood counts were performed at approximately weekly intervals. The red cell counts showed no significant variation from those of the control period. One monkey, No. 1, showed a definite leukopenia. This animal, at autopsy, exhibited widespread nodular lesions, even though all animals were tuberculin negative at the outset of the experiment. The white counts in the other 4 animals remained in the normal range. During the period of treatment 3 of the 5 monkeys showed a fluctuating, but definite eosinophilia. In contrast with the normal data reported by Downey[4] all animals during both the control and treatment periods showed a lymphocytosis.

Urine samples were collected in metabolism cages and were, therefore, subject to errors of contamination. Nevertheless, it is of interest to note that the urine samples were negative for albumin and sugar during the control period, but after the animals had been on test for 3 weeks, albumin appeared in the urine of 4 of the 5 monkeys. Sugar appeared in the urine of all 5 animals in amounts graded qualitatively from 1 to 3 plus.

Blood sugar (71 and 62 mg %) and non-

[4] Downey, H., *Handbook of Hematology*, P. B. Hoeber, New York, 1938.

protein nitrogen (31 and 32 mg %) analyses in 2 monkeys just prior to the termination of the experiment were within the normal range, (sugar 60-80 mg % and non-protein nitrogen 30-40 mg %). Approximately 2 to 5 hours after the last dose of the drug just before the animals were sacrificed, samples of blood and spinal fluid were withdrawn and analyzed for their bacitracin content. The blood concentrations were 6.6, 0.75, 0.18, 8.1, and 6.6 units per cc and the spinal fluid concentrations were 0.34, .009, .08, and .34, and .34 units per cc, respectively, indicating that the antibiotic does not readily pass the blood brain barrier in the normal monkey.

Gross examination of the organs revealed in monkey No. 1, firm grey nodules 1 to 5 mm in diameter in the lungs, liver, spleen, kidney, and heart. The kidneys of the other monkeys were moderately enlarged and edematous. The cut surface lipped over the capsular edge. The capsule stripped with ease, revealing a smooth surface. The kidneys exhibited a diffuse deep yellow color with an orange tint. The muscle at the site of injection was edematous and, in places, there were small areas of necrosis. No other gross abnormalities were noted.

Microscopic examination of the kidney in the monkeys disclosed prominent epithelial elements in the glomeruli; and, in some cases, these contained an abundant clear cytoplasm. The cytoplasm of the convoluted tubules was eosinophilic (hematoxylin-eosin stain). There was coagulum in the loops of Henle and the collecting tubules; at times, globoid bodies were present. In the loops of Henle some of the cells were vacuolated. In the tubules of monkeys No. 1 and No. 2 necrotic cells could be found only very infrequently. No necrotic cells were found in the kidneys of any of the other monkeys. There was an occasional collection of round cells in the medulla. No excess fat could be demonstrated by Sudan stains in frozen section. Best's carmine stain revealed no glycogen.

The liver contained considerable glycogen. No cellular damage was seen. The spleen showed no significant changes. The bone marrow was hyperplastic. In the lung of monkey No. 44 there were foci of a sub-acute pneumonia. The testes showed no spermatogenesis. Since the age of the animals is unknown, this finding cannot be evaluated. The thyroid in monkey No. 1 showed slight glandular hyperplasia. The intestine in monkey No. 30 was ulcerated and showed a granulomatous reaction in the wall, and parasites resembling oxyuris were found in the lumen. In the adrenals, the reticularis was dense. The muscle at the site of injection contained collections of round cells and polymorphonuclear leukocytes which extended for a considerable distance between the muscle fibres. Small areas of necrosis were present. In monkeys No. 1 and No. 2, sarcosporidia were found.

In monkey No. 1 the nodules in the lung, liver, spleen, kidney, and heart were granulomatous and contained numerous epithelioid cells. No acid-fast bacilli or other organisms could be demonstrated in the tissues with Ziehl-Neelsen stain. The etiological factor for these lesions was not ascertained.

In contrast with the striking kidney lesions in the mouse,[1] the kidney in the rat[1] and the dog did not vary appreciably from the normal. The renal lesions in the monkey were insignificant in comparison with those of the mouse.

Additional Experiments. Since bacitracin is harvested from bacterial cultures, it was of interest to determine whether or not the crude materials at hand were anaphylactogenic. Accordingly, attempts were made to sensitize each of 6 guinea pigs with 5 mg (150 units) of bacitracin administered subcutaneously as a single dose. One month later a shocking dose of 10 mg (300 units) was administered intracardially with no evidences of anaphylaxis, but additional experiments would be required to demonstrate that none of the phenomena of anaphylaxis can be caused by bacitracin.

Bacitracin solution (lot B-100 at a concentration of 6000 units per cc of distilled water) was injected intradermally in doses of .05 and .10 cc into different areas of the

shaved abdominal skin of each of 5 rabbits with no visible reaction within the ensuing 4 days.

A solution of 1200 units of bacitracin per cc of normal saline at pH 7.0 was instilled into the conjunctival sac of rabbits and the lids were held closed for 2 to 5 minutes. Even though the residual solution was not washed out, only faint evidences of irritation were noted in each of the 5 rabbits used. The faint reddening of the conjunctiva disappeared within 4 hours in all animals.

It is to be noted that the foregoing results were obtained with relatively impure commercial bacitracin concentrates as currently produced. Somewhat different results may be obtained with pure materials.

Summary. 1. Following prolonged daily administration of crude bacitracin concentrates in dogs and monkeys, no significant changes in blood morphology were observed. 2. Injection of bacitracin solution (6000 units per cc) into the shaved abdominal skin, and instillation of bacitracin (1200 units per cc) into the conjunctival sac of the rabbit caused little irritation. 3. Repeated intramuscular injection of 1000 units of bacitracin per kg 3 times each day for 23 days into the dog produced local induration. 1500 units per kg twice a day for 45 days in the same area in monkeys produced both induration and small areas of necrosis. 4. In the dog, urine samples remained negative for sugar and albumin while in the monkey, sugar and albumin appeared in the urine as the animals continued on test. 5. Large doses of bacitracin, approximating the LD_{50}, produced damage to the renal tubules, with tubular necrosis in the mouse. In the rat and the dog, the lesions were, insignificant, while in the monkey, necrotic cells were found in two instances only, and then in comparatively insignificant numbers as contrasted with the mouse.

16157

Comparison of Intestinal Lengths and Peyer's Patches in Wild and Domestic Norway and Wild Alexandrine Rats.*

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The two most common species of rats in the world, the Norways and the Alexandrines or roof rats, have very different resistance to poisoning with alpha naphthyl thiourea (ANTU) and show very different symptoms. Alexandrines have an LD_{50} of 250 mg/kg which is more than 30 times as high as the LD_{50} of the Norways, 6.9 mg/kg.[1] Furthermore, in the latter species ANTU produces a marked pulmonary edema and pleural effusion, while in the former species it causes no detectable change in the lungs or in any other organs.[2] At present these species differences in toxicity and physiological effects remain unexplained. The observation[2] that herbivorous animals such as rabbits, guinea pigs, meadow mice, prairie dogs, ground squirrels, and monkeys have a high resistance to ANTU poisoning and show no lung effects, while carnivorous or omnivorous animals such as dogs and pigs do show a marked pulmonary edema and pleural effusion and a relatively low resistance, may throw some light on this problem,† The Alexandrine rat superficially resembles the Norway rat; often inhabits the same houses and buildings; and in general

* This work was begun under a grant from the Rockefeller International Health Board and completed under a grant from the Public Health Service.

1 Dieke, S. H., and Richter, C. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 22.

2 Richter, C. P., *J. A. M. A.*, 1945, **129**, 927.

also lives in close association with man and man's food. It may, however, belong to the herbivorous group of animals listed above, or at least when compared to the Norway it may have a more herbivorous diet. Many workers who have observed the Alexandrine rat in the field believe that it does have a predominately herbivorous diet.

To throw some light on these questions we have now undertaken several comparative studies on Alexandrine and Norway rats. Some of these studies are anatomical, concerned with physical characteristics, while others are behavioristic, concerned with dietary selections. The present report deals with a comparison of the gross anatomical characteristics of the intestinal tracts of these two species of rats; the lengths of the small and large intestines and the number of Peyer's patches on the small intestines. Intestinal lengths were selected for examination since it is well known that in general herbivores have longer large intestines than do carnivores or omnivores, and often shorter small intestines.[3,4] The laboratory or domestic Norway differs anatomically and physiologically in a number of ways from its wild Norway counterpart, so it was also included in the present study. Our observations were limited to the wild form of Alexandrine rats because these rats have not been domesticated.

Methods. The wild Norway rats were trapped in Baltimore and surrounding farms by the City Rodent Control Department and the Rodent Ecology Division of the School of Hygiene of the Johns Hopkins University. Almost all of the wild Alexandrine rats came from ships in the Baltimore harbor where they had been killed by fumigation.‡ Some of the domestic Norways came from our laboratory colony, descendants of a colony of Wistar rats established 20 years ago. Others came from the Carworth Farms, and still others from Dr. E. V. McCollum's colony. The wild and domestic Norways were killed with ether. Within one hour afterwards the intestinal tract was removed, freed from all connections, washed, and evacuated. It was suspended over a strip of cross-section paper and then both were laid flat on a table. The location of the pyloric and cecal ends of the small intestine and the cecal and rectal ends of the large intestines were marked on the paper and measured. The location and size of the Peyer's patches were also indicated by dots on the chart paper. The size of the dots was made to conform roughly to the size of the Peyer's patches. For the Alexandrine rat the technique was much the same except that the rats were killed with cyanide gas and were not always autopsied within the first few hours after death; in a few instances they were not autopsied until 18 hours post-mortem. When not autopsied immediately they were kept in a refrigerator. To give a clear definition to the Peyer's patches, particularly in very young rats, the intestines were in some instances filled with a 1% solution of hematoxylin which stained all of the walls of the intestines except the intestinal patches; in other instances the rats were given olive oil by stomach tube several hours before they were killed.

Observations were made on 54 wild Norways; 58 domestic Norways; and 50 wild Alexandrines. Each group contained approximately the same number of males and females.

Results. Intestinal Lengths. Fig. 1 summarizes the results of the observations made on intestinal lengths of the two strains of Norway rats. The ordinates give intestinal length in centimeters; the abscissae body weight in grams. The rats were fairly evenly distributed over the total body weight range. The heaviest wild Norway that we could get for this study weighed 499 g; the heaviest

† Cats, which definitely belong to the carnivorous group of animals, show all of the lung effects that are found in rats, pigs, and dogs, but they do not have as low an LD_{50}, at least when they receive the ANTU by stomach tube. Their ready ability to vomit may explain the higher LD_{50}. We have not determined the parenteral LD_{50}.

3 Buddenbrock, W. von, *Grundriss der vergleichenden physiologie,* Berlin, 1928, p. 650.

4 Dukes, H. H., *The Physiology of Domestic Animals,* 4th edition, Comstock Publishing Co., 1937.

‡ Dr. M. F. Haralson, in charge of the Quarantine Station of the City of Baltimore, kindly supplied us with these rats.

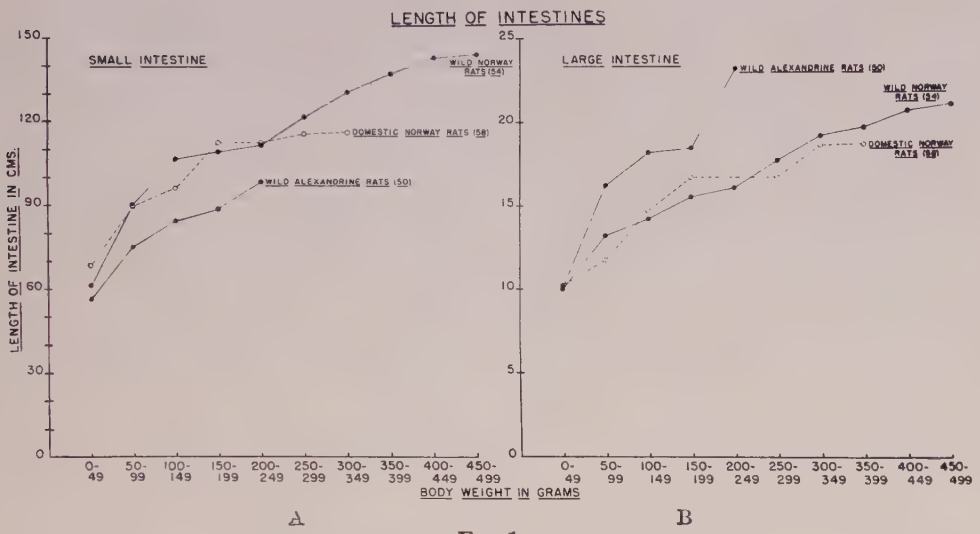

FIG. 1.

Graph showing the relation between the length of the gastro-intestinal tract and body weight. 1A. Small intestines. 1B. Large intestines.

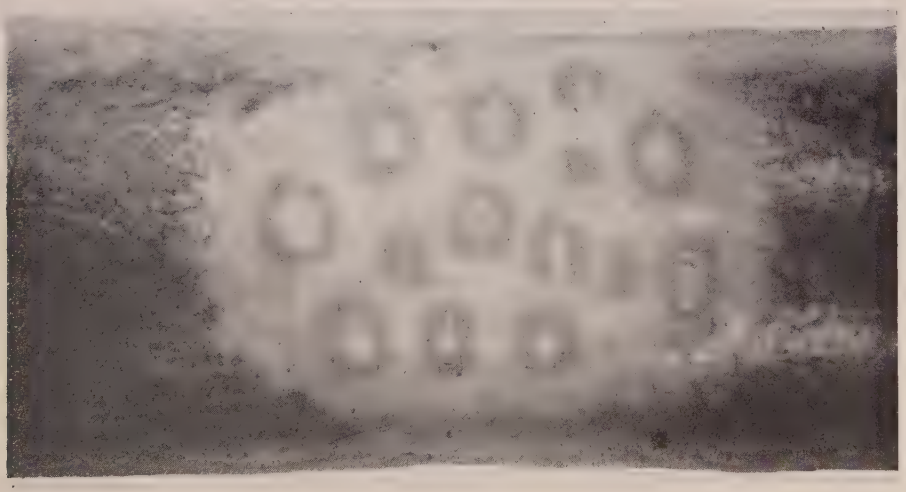

FIG. 2.

Photograph of typical Peyer's patch. Intestine filled with hematoxylin.

domestic Norway 349 g; and the heaviest Alexandrine 248 g.

Fig. 1a shows that the Norway rats, both wild and domestic strains, had definitely longer small intestines than did the wild Alexandrines. In the 200-249 g range the small intestines of the Norways had an average length of 113 cm while those of the wild Alexandrines averaged only 98 cm. The small intestines of the domestic Norway were of essentially the same length as those of its wild congener in all weight ranges investigated up to 300 g.

Figure 1b shows that the reverse relationship holds for the length of the large intestines. The wild Alexandrines had definitely longer large intestines than did either the wild or domestic Norways. For the 200-249 g weight group the length of the large intestine averaged 23.0 cm for the wild Alexandrines, and only 16.0 and 16.5 cm respectively for the wild and domestic Norways. Here again the

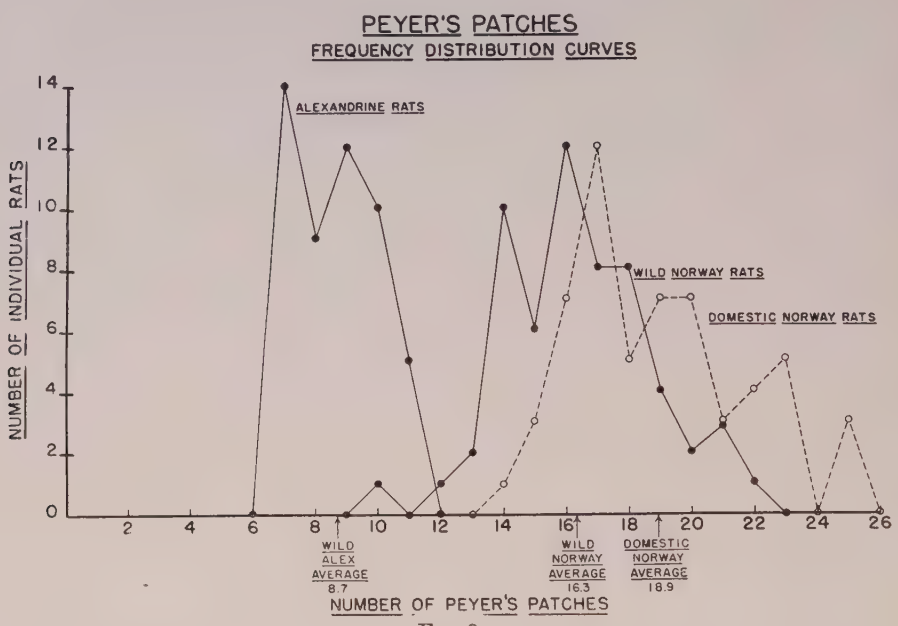

FIG. 3.
Chart showing frequency distribution curves of Peyer's patches.

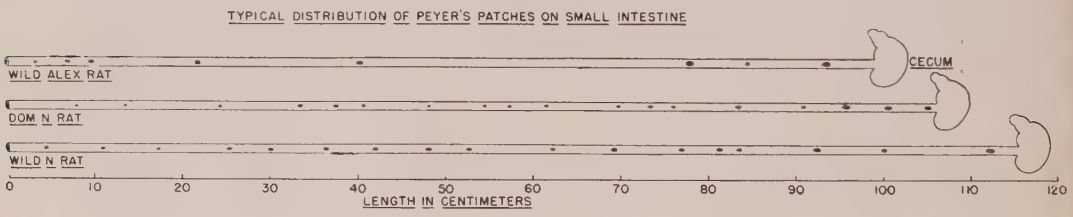

FIG. 4.
Drawing of 3 intestinal tracts from wild Alexandrines, domestic Norways, and wild Norways respectively, showing distribution of Peyer's patches.

wild and domestic Norways had essentially the same intestinal lengths in all weight groups.

Peyer's Patches. Fig. 2 shows a photograph of a Peyer's patch which is fairly typical for the two Norway strains and for the wild Alexandrines. The intestine of this rat was filled with the hematoxylin solution. This patch contained 13 follicles.

Fig. 3 gives the frequency distribution curves for the number of Peyer's patches found in the 3 types of rats. It shows that the Norway rats had many more patches than did the Alexandrine rats. For the Alexandrines the average number and its standard error were 8.7 $\pm$ 0.19, for the wild Norway 16.3 $\pm$ 0.31, and for the domestic Norway 18.9 $\pm$ 0.37. The curves for the wild Alexandrines and Norways showed almost no overlap at any point. Fig. 4 shows a typical distribution of Peyer's patches on the small intestines of a wild Alexandrine, domestic Norway and wild Norway respectively. The sizes of the patches are drawn roughly to scale. In the wild Alexandrine most of the patches were usually found near the two ends of the intestine, while in the 2 Norway types the patches were distributed at fairly regular intervals throughout the entire length of the intestines. For all the 3 types of rats the patches tended to become much larger in the lower third of the intestines. We were not able to establish any relationship between number of Peyer's patches and age or sex. At an age of approximately 15 days when the patches first became visible the number pres-

ent was comparable to that found in adult rats.

Discussion. The results show that compared to the omnivorous or carnivorous Norway rats, the Alexandrine rat has a definitely shorter small intestine and a longer large intestine. Thus, according to these findings, the Alexandrine rat either belongs to the herbivorous group of animals, or at least when compared to the Norway it must have a much more herbivorous diet. It should thus be able to utilize larger amounts of cellulose material.

These findings in themselves do not prove that the Alexandrine rat is an herbivore. They will have to be substantiated by the results of dietary selection studies. They do indicate, however, that the high resistance of the Alexandrine rat to ANTU poisoning and its lack of any lung effects may depend on some factors which the herbivorous animals have in common. The observation that the Alexandrine rats have only about half as many Peyer's patches as do the Norways has an interest quite apart from any light that it might throw on the question whether or not the Alexandrine rats belong to the herbivore. The Peyer's patches on the intestines have been counted in a number of different animals, horses, cows, sheep, pigs, dogs, cats and rabbits. They range in number from 2-4 for cats to 180-320 for horses.[5] Kelsall[6] recently counted the number of patches in different strains of mice and found that the means varied from 6.3 in the C57 black strain to 10.7 in the C3H strain, while intermediate groups including strains C, Swiss, and dba, had 8-9 Peyer's patches. Clearly, in number of patches the mice show a much closer relationship to the Alexandrine rats (which had an average of 8.7 patches) than they do to the Norways with an average of 16-18 patches.

Mice as well as the Alexandrine rats appear to prefer an herbivorous diet. They live largely on grain, flour, and cereal. This suggests that a correlation may exist between the dietary habits and the number of Peyer's patches, or indirectly the total amount of lymphoid tissue in the body. An association of a lower amount of lymphoid tissue with an herbivorous diet however would not agree with the well-known fact that in general, herbivorous animals have more lymphoid tissue than do carnivorous animals.[7] This discrepancy may result from the assumption that the number of Peyer's patches bears a direct relation to the total amount of lymphoid tissue in the body. Although in general appearance, behavior and habits, mice appear to be more closely related to Alexandrine rats than they do to Norways, they have a lower resistance to ANTU poisoning than do Alexandrines, and also show pulmonary edema and pleural effusion.

Kelsall[6] reported a direct correlation "between the amount of intestinal lymphoid tissue, as measured by the number of Peyer's patches, and the incidence of spontaneous mammary tumors." She reported that in the strain of mice which had only an average of 6.3 patches the incidence of tumors was very low, while in the breeding females of those with an average of 10.7 patches it was 95%. On this basis the Norway rats with their much higher number of Peyer's patches should exhibit a higher incidence of tumors than do the Alexandrines. Our results show that domestic Norway rats have more Peyer's patches than do the wild Norways. We have no explanation for this finding. Previous experiments have revealed other differences between the wild and domestic rats.[8–12] In this connection one of the most interesting differences is that the domestic rats have much smaller adrenal glands.[12] This recession of

5 Patzelt, V., *Handb. der Mikro. Anat. des Mensch.*, Julius Springer, Berlin, 1936, **5**, 218.

6 Kelsall, M. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 423.

7 Ehrich, W. E., *Ann. New York Acad. Sc.*, 1946, **46**, 823.

8 Dieke, S. H., and Richter, C. P., *J. Pharm. and Exp. Therap.*, 1945, **83**, 195.

9 Fish, H. S., and Richter, C. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 352.

10 Griffiths, W. J., Jr., *Science*, 1944, **99**, 62.

11 Griffiths, W. J., Jr., *Am. J. Physiol.*, 1947, **149**, 135.

12 Rogers, P. V., and Richter, C. P., in press.

the adrenals might have resulted in an increase in lymphoid tissue, since it is well known that after removal of the adrenals the thymus and other lymphoid tissue hypertrophies.

Summary. 1. Alexandrines or roof rats (*Rattus rattus*) have shorter small intestines and longer large intestines than do Norway rats (*Rattus norvegicus*). 2. The intestines of Norway rats have about twice as many Peyer's patches on the average as do those of Alexandrine rats, averaging 16-19 as compared to 9. 3. The intestines of domestic Norway rats have more Peyer's patches than do those of wild Norways. 4. Peyer's patches first become visible when the rats reach an age of 15 days. The number does not appear to change with age.

16158 P

Effect of Interval Between Hypophysectomy and Injection of Gonadotrophin on Ovulation in the Rat.*

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Hertz and Meyer[1] have shown that if young adult female rats are hypophysectomized during the first 2 hours of proestrus ovulation is prevented during the subsequent 46-hour period. It was concluded that a hypophyseal hormone (LH) is required for ovulation in the rat and that the quantity required is secreted subsequent to early proestrus.

The work reported here was undertaken to determine how long after hypophysectomy mature follicles retain their ability to ovulate in response to a single intravenous injection of human chorionic gonadotrophin, and is preliminary to a series of experiments designed to study the hormonal requirements for normal development of follicles and ova just prior to ovulation.

Method and Materials. The 72 rats employed in this experiment were 3- to 5-month-old virgin females of the Sprague-Dawley strain.

All hypophysectomies were carried out during the first 3 hours of proestrus using virtually the same technique described by Hertz and Meyer.[1]

The intravenous injections of the purified human chorionic gonadotrophin, prepared essentially by the method of Gurin, *et al.*,[2] were given in one of the tail veins with the exception of a few injections which were made into the heart. Fifty and 100 γ doses of the dry powder in an alkaline saline solution at a concentration of 100 γ/cc were given but since the results for the 2 doses were comparable, they have been combined in Table I. The timing of the injections in relation to hypophysectomy is also shown in this table.

Autopsies were carried out 30 to 50 hours after the injections allowing ample time for all the ova which had ovulated to reach the oviduct. The sella was examined with magnifying spectacles to determine if the hypophysectomy had been complete. The ovaries and oviducts were removed and fixed in FAA or Zenker's solution.

Serial sections of one ovary and oviduct from each rat were made and stained with hematoxylin and eosin. The sections were then examined for the presence of ova in the ovi-

* Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1 Hertz, Roy, and Meyer, R. K., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 71.

2 Gurin, S., Bachman, C., and Wilson, D. W., *J. Biol. Chem.*, 1939, **128**, 525.

TABLE I.
Effect of Varying the Interval Between Hypophysectomy and Gonadotrophin Injection on Ovulation in the Rat.

Hrs from hypophysectomy to injection	No. of rats hypophysectomized	No. of rats ovulated
0	6	6
2	6	5
4	9	8
6	8	7
8	12	11
10	14	6
12	8	1
19	6	0
24	5	0

duct, newly formed corpora lutea, and preovulatory follicles.

Results and Discussion. The results as summarized in Table I show that in a high percentage of the animals tested the mature follicles retain their ability to ovulate in response to an intravenous injection of chorionic gonadotrophin provided the injection is made within 8 hours after the hypophysectomy. If the interval between hypophysectomy and injection is extended to 10 or more hours, the percentage of animals which will respond declines rapidly with no animals responding after 12 hours.

The data show that the pituitary is necessary for maintaining the mature follicles found in early proestrus so that they will ovulate in response to a gonadotrophic stimulus. They also indicate that after hypophysectomy the amount of the pituitary hormones responsible for the maintenance drops rapidly to a level in the blood below that required to keep the follicles in a condition where they can be ovulated.

It was found that the follicles in the ovaries of the rats injected 8 to 12 hours following hypophysectomy which failed to ovulate often showed a marked degree of luteinization, but when the interval was extended to 19 hours or more luteinization did not occur.

Summary. After an interval of about 10 hours following hypophysectomy in early proestrus, the mature follicles in the ovary of the rat become refractory to a single intravenous injection of human chorionic gonadotrophin but the ability of the follicle to luteinize is retained for a longer time.

16159 P

Statistical Evaluation of Growth Curves.

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In an article with the above title which appeared in this journal,[1] Weil compares the growth curves of groups of experimental animals subject to different treatments. Five treatments are compared against each other and a control, 30 rats are used for each treatment and the weight of each rat is determined at weekly intervals. Weil proposes a method of analysis in which he constructs a frequency distribution for each treatment taking all the weights of the rats over the period of the experiment and then applies the chi-square test to examine differences between those frequency distributions for the various treatments.

This is an oversimplified method of analysis and not a valid one. The fallacy lies in the tacit assumption that all the observations within each of the frequency distributions are independent. Although the 30 rats in each group are independent, the repeat weighings on each rat are certainly not so. In practically all biological work, the main source of variability lies between animals;

1 Weil, C. S., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 468.

repeat determinations per animal will result in more precise measurement for the individual animals, but will not reduce the effect of the basic variability between the animals. Applying the chi-square test in the way proposed by Weil may grossly overestimate the significance of the differences between the treatments.

There are valid methods of comparing growth curves of the type considered by Weil. These are indicated in broad outline in this note and a more detailed account with numerical examples will be published elsewhere.

In his paper, Weil considers the t-test applied to the weights after a given time, *e.g.* weights after 12 weeks. Provided the apportionment of the animals between the groups has been carried out in a strictly random manner, this method of test is valid. It may not be the best since it does not make use of the earlier weighings. Some improvement would probably result if the final weights are corrected for the variations in the initial weight of the rat (or any other characteristic which may be correlated with the final weight). The method to use here is the covariance method discussed by Fisher.[2]

A more complete method would be to fit regression lines, such as by the method of least squares, to the growth curve of each rat using, if required, a simple transformation to the time scale and weight scale in order to produce a simple curve. We may apply the method of the previous paragraph to estimates of the weight of rat obtained from the fitted lines at any given time. We could go further if desired and assess the significance of the differences between the constants of the fitted curves. For example, in the case of the slope of the curve, we adjust this slope (if required) for the estimated initial weight of each rat and calculate the significance of the effect of treatments on the adjusted slopes by Fisher's method already referred to. If the growth curves have been transformed into straight lines, this would represent a complete analysis.

Sufficiently approximate results may often be obtained from curves fitted by eye. We could compare any property of the growth curves between groups, *e.g.*, rate of growth at any given time, increase in weight between any given times, etc.

When the growth curves cannot be conveniently transformed into straight lines, these methods may have the disadvantage of not measuring the overall differences between the growth curves. A satisfactory method would be to read off from the fitted curves (by eye or by calculation) the estimated weights at the initial time and at two other times chosen to give a fairly adequate description of the growth curves. We then apply the method of "discriminant function analysis"[2] to these pairs of weights. This gives the maximum discrimination between the treatments and at the same time, furnishes a satisfactory test of the significance of the differences between the treatments. When it is desirable to correct for variations in the initial weight of the rats, we can apply this correction and the discriminant function analysis simultaneously.

With, if necessary, the use of a simple transformation for either or both of rat weight and time, it is nearly always possible to give an adequate description of the growth curves from 2 or 3 points. In rare cases a fourth point might be necessary. The discriminant function analysis can be extended quite readily to accommodate another point.

[2] Fisher, R. A., *Statistical Methods for Research Workers*, Ninth Edition, Oliver & Boyd, Ltd., Edinburgh and London, 1944.

16160 P

An Experimental Study of the Cerebral Coproporphyrin in Rabbits.*

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Klüver's discovery of the presence of small amounts of coproporphyrin in the central nervous system of warm-blooded animals[1,2] raised the question whether there might be a relationship between this porphyrin and the increased urinary coproporphyrin in conditions such as lead and arsenic poisoning, which are characterized both by injury of the nervous system and by marked coproporphyrinuria. It is well known that in heavy metal and chemical poisoning the excess urinary coproporphyrin is the type III isomer.[3] Preliminary studies by Klüver,[4] and in this laboratory, with the differential precipitation, or "fluorescence quenching" technique[5,6] indicate that the coproporphyrin of the nervous system is likewise the type III isomer. Hence it seemed desirable to determine the coproporphyrin concentration in the brains of rabbits suffering from acute lead poisoning.

Hitherto, quantitative data on the coproporphyrin of the central nervous system have not been reported, nor has a quantitative method of determination been described. The method devised for use in the present study combines certain features of procedures previously described for urinary coproporphyrin[6] and erythrocyte protoporphyrin.[7] The procedure was briefly as follows: The entire rabbit brain was ground in a mortar, washed repeatedly with physiological saline solution to remove most of the blood, and then mixed with 10 ml of glacial acetic acid and 100 ml of acetone. The mixture was allowed to stand overnight with repeated shaking. It was filtered through cheesecloth and the residue was ground with additional amounts of glacial acetic acid and acetone, and finally with ethyl acetate.† The residue was pressed as dry as possible. The combined filtrate was mixed with an equal volume of distilled water and extracted 4 times with ethyl acetate. The latter was extracted 4 times with 15 ml portions of 10%HCl. The acid extract was made negative to Congo red paper by addition of saturated aqueous sodium acetate solution, and the solution was extracted 4 times with ethyl acetate after a few ml of glacial acetic acid had been added. The combined ethyl acetate extracts were washed with water and

TABLE I.
Cerebral Coproporphyrin in Normal Rabbits.

No.	Wt of brain in g	γ of coproporphyrin (Total content)	γ/g brain
1	8.1	.3	.037
2	9.1	.225	.025
3	8.5	.57	.067
4	8.3	.30	.036
5	8.0	.27	.034
6	7.6	.345	.045
7	9.0	.225	.025
8	8.7	.225	.026
9	8.0	.30	.038
10	7.7	.18	.023
		Avg	.0356

† More recently it has been found advantageous to grind and extract the brain with glacial acetic acid and ethyl acetate (1:10), on a sintered glass filter; several such extractions obviate the necessity of preliminary extraction with acetone and the period of standing in contact with it. (C. J. W.)

* Aided by grants from the John and Mary R. Markle Foundation, New York City, and the Medical Research Fund of the Graduate School, University of Minnesota.

1 Klüver, H., *J. Psychol.*, 1944, **17**, 209.

2 Klüver, H., *Science*, 1944, **99**, 482.

3 Watson, C. J., and Larson, E. A., *Physiol. Rev.*, 1947, **27**, 478.

4 Klüver, H., personal communication.

5 Schwartz, S., Hawkinson, V. E., and Watson, C. J., *Science*, 1946, **103**, 338.

6 Schwartz, S., Hawkinson, V. E., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, **168**, 133.

7 Grinstein, M., and Watson, C. J., *J. Biol. Chem.*, 1943, **147**, 675.

TABLE II.
Urinary and Cerebral Coproporphyrin in Lead-Poisoned Rabbits.

No.	UCP* in γ/day at time of killing	Wt of brain in g	γ of coproporphyrin (total content)	γ/g brain
1	12.6	7.5	.3	.04
2	16.0	9.1	.48	.052
3	20.0	8.0	.195	.024
4	3.7	8.5	.30	.035
5	11.5	9.3	.195	.021
6	19.8	9.2	.345	.037
7	43.0	8.3	.15	.018
8	58.0	8.6	.195	.023
9	49.0	8.0	.345	.043
10	44.0	9.1	.345	.038
11	15.0	7.7	.30	.040
12	26.0	8.0	.405	.05
			Avg	.035

* UCP = total urinary coproporphyrin.

extracted 4 times with 2-3 cc portions of 1% HCl. This was washed with $CHCl_3$, separated and filtered. The determination of total coproporphyrin was then made on the 1% HCl extract in the Klett fluorophotometer, as previously described.[6] The concentration and individual content was determined for the brains of 10 normal rabbits, data for which are given in Table I.

Twelve rabbits were poisoned with lead acetate, the first 6 receiving 50 mg per kilo, the second six 100 mg per kilo, in a single intraperitoneal injection. The animals were killed 7-10 days later. The analytical data are given in Table II. The urinary coproporphyrin was determined by the method of Schwartz and associates[6] which gives an upper limit of 5 γ per day for normal rabbits.

No increase in the brain coproporphyrin occurred following the acute lead poisoning. Thus it is evident that the present study does not provide evidence of a relationship between the coproporphyrin content of the brain and that of the urine which was much increased. The possibility cannot be excluded that an accelerated formation and release of coproporphyrin by the brain could have maintained the actual concentration at a constant level. Other studies are in progress in which various substances affecting the nervous system are being used, both in acute and chronic experiment.

Summary and Conclusions. 1. A method is described for the quantitative determination of coproporphyrin in the brain. 2. The concentration and total content of the coproporphyrin of normal rabbit brains was compared with that of brains from rabbits, in which acute lead poisoning was induced. No variation was observed.

16161 P

Coronary Sinus Catheterization Technique for Studying Coronary Blood Flow and Myocardial Metabolism *in vivo*.

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Recent reviews stress the need for a method of studying the coronary circulation in intact animals, as well as in man.[1,2] Harrison and coworkers cannulated the coronary sinus of intact morphinized dogs,[3] with a modified Morawitz brass balloon cannula.[4] A less traumatic technique of intravenous catheterization of the coronary sinus of intact dogs is presented in this report, using the soft Forssman catheter,[5] as modified by Cournand.[6] Application of this method in studying myocardial metabolism *in vivo* will be reported elsewhere,[7,8] particularly the measurement of coronary blood flow by the nitrous oxide method, developed by Kety and Schmidt for measuring cerebral blood flow.[9]

The catheter,[6] (size 7-9, reasonably stiff but without stylet), is inserted through the dog's external jugular vein, under light nembutal anesthesia. Fluoroscopically, in the right anterior oblique position,* a triangular area of lung with the following boundaries is visible: (1) the anteromedial border of the inferior vena cava, (2) the posteroinferior cardiac border, and (3) the diaphragm. The coronary sinus ostium lies just anteromedial to the superior corner of this triangle which marks the junction of inferior cava and right auricle, posteroinferior to the tricuspid valve. The catheter is first passed into the inferior cava, then withdrawn just inside the auricle. As the tip is shifted anteromedially, with repeated gentle thrusts toward the ostium, the catheter will eventually enter the coronary sinus, (Fig. 1). The tip often passes further in the same direction, superiorly and to the left along the auriculo-ventricular groove, past a delicate valve into the great cardiac vein. Sometimes the catheter enters the middle cardiac vein, or more rarely the first posterior vein of the left ventricle, and passes along the posteroinferior septal surface toward the apex.

Evidence of successful coronary sinus catheterization includes: (1) the typical fluoroscopic position of the catheter,† (2) withdrawal of very dark venous blood which shows an extremely low oxygen content, markedly lower than in mixed venous blood, (Table I), and (3), in some cases, autopsy with the catheter still inserted in the sinus.

The coronary sinus of 30 dogs, weighing 28-75 lbs., has been successfully catheterized 55 times, with as many as 7 procedures on the same dog at monthly intervals. Three attempts in small dogs were failures. Postoperative recovery was prompt, except in those intentionally sacrificed. No post-

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3 Harrison, T. R., Friedman, B., and Resnick, H., Jr., *Arch. Int. Med.*, 1936, **57**, 927.

4 Morawitz, P., and Zahn, A., *Zentralbl. f. Physiol.*, 1912, **26**, 465.

5 Forssman, W., *Klin. Wchnschr.*, 1929, **8**, 2085.

6 Cournand, A., *Fed. Proc.*, 1945, 207.

7 Eckenhoff, J. E., Hafkenschiel, J. H., Harmel, M. H., Goodale, W. T., Lubin, M., Bing, R. J., and Kety, S. S., in press.

8 Goodale, W. T., Lubin, M., and Banfield, W. G., in press.

9 Kety, S. S., and Schmidt, C. F., *Am. J. Phys.*, 1945, **143**, 53.

* Terminology, as in *man*.

† Diodrast (3,5-diiodo-4-pyridone-N-acetic acid and diethanolamine) was occasionally injected forcibly to outline the coronary venous system fluoroscopically, but sometimes caused local myocardial necrosis and hemorrhage. Forcible injection of any fluid against the coronary venous stream may be hazardous.

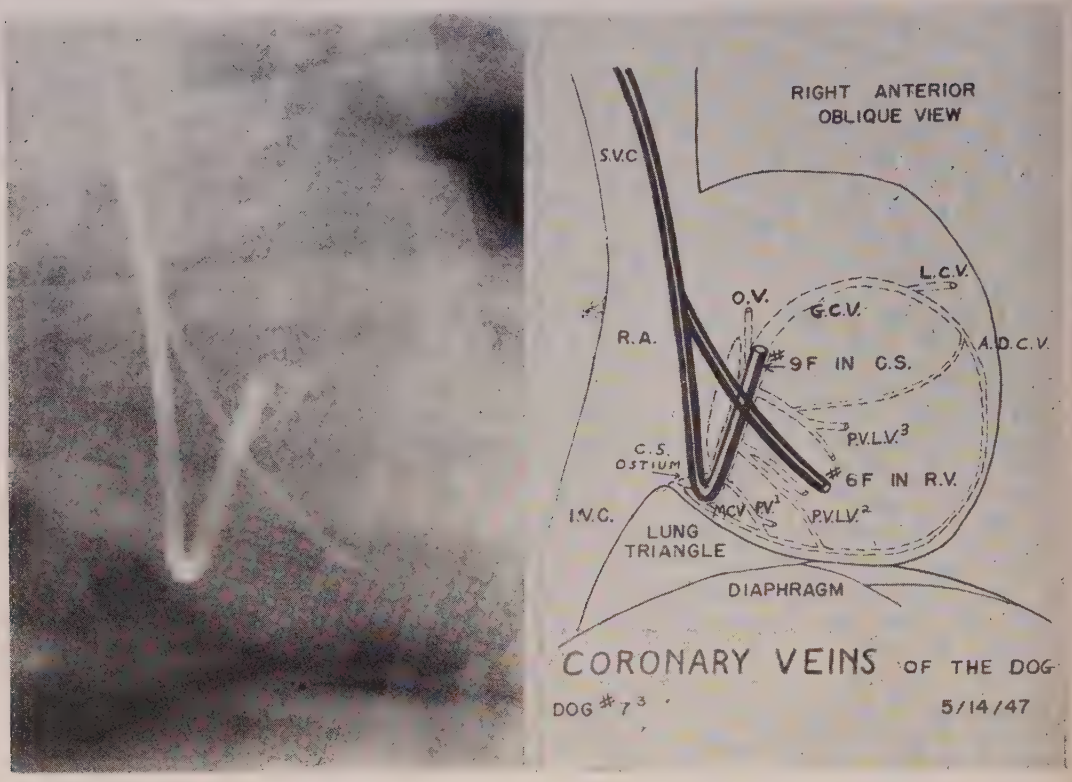

FIG. 1.

Large No. 9 catheter is inserted 3-4 cm into coronary sinus (C.S.), with small No. 6 catheter passing through tricuspid valve (— · — · —), into right ventricle (R.V.). The coronary sinus venous system, as found at autopsy, is sketched in as follows: middle cardiac vein, MCV; posterior veins of the left ventricle, PV_1, $PVLV_2$, and $PVLV_3$; oblique vein of Marshall, OV; great cardiac vein, GCV; left circumflex vein, LCV; anterior descending coronary vein, ADCV. The extensive veno-venous anastomoses between the major veins draining the left ventricle are illustrated. (The venous drainage of the right ventricle is largely independent of the coronary sinus system, through the Thebesian, anterior, and other small cardiac veins.)

Metabolic Observations: 19 Aug., 1947; Oxygen content of blood from coronary sinus, 3.7 vols. %; pulmonary artery, 14.8 vols. %; femoral artery, 18.2 vols. %, 94% saturated. Coronary flow, 71 cc/min/100 g of left ventricle. Myocardial oxygen consumption, 10.6 cc/min/100 g. Systemic blood flow, 168 cc/kg body wt/min. Weight of dog, 31 kg. Heart weight at autopsy, 240 g. Mean arterial blood pressure, 115 mm Hg. Work of heart, $7/6 \cdot QR = 503$ kg/hr. Cardiac efficiency, 16%.

Pathology: This dog was catheterized 7 times in 5 months, with a total of over 16 hours of actual insertion of the catheter in the coronary sinus, with a second catheter in the pulmonary artery or right ventricle. Autopsy 7 weeks after the last procedure showed a normal heart except for slight subendocardial fibrosis in the right auricle and medial tricuspid valve leaflet.

operative local or systemic infection, or other clinical complications attributable to catheterization, were encountered.

Thirty autopsies following coronary sinus catheterization often showed small mural thrombi and subendocardial hemorrhages in the coronary sinus and right auricle. Gross lesions were rarely found when the dog was sacrificed immediately after catheterization, but often when sacrificed at least 24 hours later. Minimal subendocardial fibrosis in the right auricle was the only finding in 4 of 5 dogs sacrificed 3 to 6 weeks after catheterization.

Mural thrombi and subendocardial hemorrhages, however, were also found in the right auricle, on the right ventricle and on the tricuspid and pulmonary valves after catheter-

TABLE I.
Oxygen Content of Blood Samples.[15]

	Oxygen content vol. %					
	No. of observations	Mean value	S.E.*	S.D.†	C.V.‡	Extreme range
Coronary sinus	44	3.8	.16	1.04	27.7	2.4- 8.3
Pulmonary artery or rt. ventricle	30	12.5	.72	3.89	31.5	9.5-15.3
Femoral artery	48	16.9	.25	1.75	10.3	14.5-22.6

* Standard error of the mean.
† Standard deviation.
‡ Coefficient of variation.

izing the pulmonary artery or right ventricle.‡ The auricular lesions were more frequent and more severe than those found after passing the catheter only into the coronary sinus.

In 3 cases, lesions obviously peculiar to coronary sinus catheterization were found: 2 of coronary venous thrombosis and one of gross hemorrhage into the myocardium drained by a catheterized vein. These were perhaps related to prolonged insertion of a large catheter beyond the sinus into the great cardiac vein, or to forceful reinjection of fluid through the catheter. Such lesions have not been found in 11 control experiments in which precautions were taken, including the gentle insertion of a No. 7-F catheter only 1-2 cm into the coronary sinus for only 60 minutes. With these precautions, coronary sinus catheterization appears to be actually less hazardous than catheterization of the right ventricle or pulmonary artery of the dog by our technique.

In man, several thousand well-controlled intracardiac catheterizations have been performed without cardiac damage apparent even in numerous autopsied cases.[6,10–14] Species peculiarities and differing techniques may well explain the occurrence of lesions in dogs, where none have so far been found in man. The present technique, however, is being applied to current studies of the coronary circulation in man only with the precautions which prevented significant myocardial and coronary venous damage in dogs.

Summary. Coronary sinus catheterization technique has been developed in intact dogs in order to study coronary blood flow and myocardial metabolism, and to evaluate the safety and practicality of a similar procedure in man. The pathological findings, among 30 autopsies following the procedure, have been discussed.

The authors are very much indebted to Drs. S. S. Kety, C. F. Schmidt, and R. J. Bing for their help and advice, and to W. P. McShane, R. C. Johnson, Miss Alice Willis, Miss Sarah Bederman, and Mrs. Pauline Wilson for skillful technical assistance.

‡The occurrence of endocardial lesions in dogs after catheterizing the pulmonary artery by this technique, has been recently confirmed, although with less frequency and severity than in the present series (Hellems, H. K., Haynes, F. W., Fanger, H., and Dexter, L., personal communication, 1947).

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12 Bing, R. J., personal communication.

13 Dexter, L., personal communication.

14 Cournand, A., personal communication.

15 Roughton, F. J. W., and Scholander, P. F., *J. Biol. Chem.*, 1943, **148**, 541.

16162

Effect of Desoxycorticosterone on the Development of Rats Treated with Thiouracil.

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It has been shown that either adrenal cortical extracts or desoxycorticosterone acetate (DOCA) hastened the development of newborn rats.[1] Under the effects of these substances, the rats' teeth erupted earlier and their eyes opened sooner than littermate controls. The mechanism whereby this precocious development takes place is under investigation. It has been reported that thyroxine produced a qualitatively similar hastening of tooth development[2] and experiments were undertaken to determine whether the adrenal cortical action was mediated through the thyroid gland.

Thyroidectomy in the newborn is an extremely hazardous and frequently fatal operation.[3] Depression of the normal thyroid activity can be accomplished in baby rats by means of thiouracil.[4,5] The effects of DOCA on thiouracil treated rats are reported.

Method. Sixty-two one-day-old rats from 6 litters of the Sprague-Dawley strain were divided into 4 groups. Wherever there were sufficient rats the individual litters were divided so that there were at least 3 rats for each of the following experimental conditions. A total of 15 rats received daily (including Sunday) subcutaneous injections of 0.25 mg of DOCA in 0.05 ml of peanut oil. Eleven rats received similar injections of one to 4 mg of thiouracil, suspended in 0.1 ml of peanut oil. Twenty-one rats received daily injections of both drugs, and 15 rats remained as the untreated control group. Injections were usually given for about 2 weeks. Each litter of rats remained with its respective mother throughout the experiment. Daily body weights were recorded for each rat and observations of tooth development, eyelid opening, and general physical condition were made each morning and night. At the completion of the experiments, the animals were sacrificed and their thyroids were sectioned and stained with hematoxylin and eosin.

Results. All the rats tolerated the injections and handling fairly well in the newborn period. Hair growth at the site of the DOCA injections was sparse in both groups of rats receiving the drug.

Table I reveals the effects of the drugs on the eruption of the incisor teeth and opening of the eyelids. Although figures given are means of results from 6 litters, the results in individual litters were similar. DOCA invariably hastened the eruption of the teeth, stimulated the differentiation and separation of the lips from the gingiva, and hastened the opening of the eyelids. Thiouracil, in doses of one to 4 mg per day, on the other hand markedly depressed all these developmental changes. In those rats receiving injections of both DOCA and thiouracil, a mutually antagonistic effect was observed. The tooth eruption and eyelid opening in these rats was within normal limits. This was confirmed statistically in that the means of the DOCA and thiouracil groups were significantly different from that of the controls, but the mean of the group receiving the combination of both drugs was not significantly different from that of the controls.

The effects on the body weight and growth curve of one representative litter of 12 rats are shown in Fig. 1. DOCA in the dose em-

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TABLE I.
Effect of Injections of Desoxycorticosterone Acetate, Thiouracil, and Combination of Both Drugs on Teeth and Eyes of Baby Rats.

	Control 15 rats		DOCA 15 rats		Thiouracil 11 rats		Thiouracil + DOCA 21 rats	
	Mean	σ	Mean	σ	Mean	σ	Mean	σ
Age at eruption of incisors (days)	9.5	0.63	8.4	0.29	11.3	0.61	9.2	0.84
Age at opening of eyelids (days)	15.1	0.43	14.1	0.61	16.9	0.45	15.5	0.73

ployed had no effect on the body weight of rats even though the injections were continued for 23 days. Thiouracil also had little or no effect on the growth curve for the first 10 to 15 days. However, there then occurred a plateauing of body weight so that in the next week, the thiouracil treated rats showed marked stunting of growth and evidence of immaturity in activity and physical appearance. This has been described by Hughes[4] as cretinism. DOCA, despite its antagonistic effect on the thiouracil depression of tooth, mouth and eyelid development, had no effect whatsoever on antagonizing this stunting effect of thiouracil. Their sickly state was not affected by DOCA and occasionally they seemed more depressed than the rats receiving thiouracil alone. One group of rats which received both drugs for a period of 2 weeks was observed further and the weight curve was compared to that of the littermate controls. At the end of 3 weeks without injections the average weight of the rats was about 15% below that of their controls. In another litter, 2 of the 4 rats in a similar group became weak, stopped feeding and died, despite the cessation of treatment.

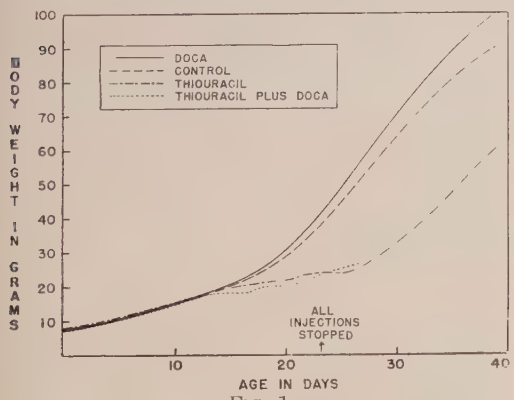

FIG. 1.

Growth curves of baby rats receiving DOCA, thiouracil, or a combination of both drugs. There are 3 rats in each group and all are littermates.

The thyroid glands of the thiouracil treated rats were grossly much larger than those of the controls. Histologically they showed enlargement and increased height of the follicle cells. The glands of the animals receiving the combination of DOCA and thiouracil were indistinguishable from those treated with thiouracil alone, whereas those injected with DOCA alone resembled the normal controls.

Comment. There is little evidence from these experiments to indicate that the precocious development produced by DOCA is mediated through the thyroid gland. Although thiouracil depresses the normal thyroid gland function, there is no certainty that it does so completely in the first few injections. If DOCA could produce its usual changes in a surgically thyroidectomized rat, a definite conclusion could be arrived at.

It is of interest, that despite the fact that thiouracil produces marked changes in the development and growth of the rats, DOCA was able to antagonize its effects on tooth eruption and eyelid opening. Whether the same phenomena can be produced by large doses of other steroid hormones and whether other effects of the thiouracil-thyroid deficiency can be corrected by these hormones remains to be studied.

Summary. Thiouracil injections into baby rats resulted in retardation of tooth eruption and opening of the eyelids. Desoxycorticosterone, when injected into thiouracil treated rats, was able to antagonize these developmental effects so that the time of tooth eruption and eyelid opening returned to normal. It had no antagonizing effect, however, on the stunting of body growth or on the histological picture of the thyroid gland produced by thiouracil.

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Urinary Excretion Studies Following the Administration of Pteroic Acid.

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A number of reports have dealt with the apparent urinary excretion of pteroylglutamic acid as measured with *Lactobacillus casei* or *Streptococcus faecalis* R. In normal subjects on "average diets," the excretion is quite low, usually in the neighborhood of 4 to 5 μg daily. The urinary excretion rises following the administration of a few milligrams of the pure substance. In one investigation, assays with *S. faecalis* R indicated that from 44 to 57% of an oral dose of 5 mg was excreted in six hours.[1]

The name pteroic acid[2] has been given to the portion of the pteroylglutamic acid molecule exclusive of glutamic acid, more specifically 4(((2-amino-4-hydroxy-6-pteridyl)methyl)amino)benzoic acid. It may be synthesized by condensing p-aminobenzoic acid with α-β-dibromopropionaldehyde and 2,4,5-triamino-6-hydroxypyrimidine.[2] Pteroic acid can replace pteroylglutamic acid in promoting growth of certain microorganisms, notably *S. faecalis* R, but it is without appreciable growth-promoting action on *L. casei*.[2] The presence of pteroic acid in the urine would therefore produce a response in the "folic acid" assay when carried out with *S. faecalis* R but would be without effect if the assay were carried out with *L. casei*. No studies have been reported describing the urinary excretion of pteroic acid following its administration and such an investigation was the subject of the present study.

Experimental. Pteroic acid was synthesized as described above* and was obtained in a state of approximately 90% purity as estimated by chemical assay[3] and extinction coefficient measurements. The compound was administered as a solution of the monosodium salt to normal adult male subjects. The urine was collected under toluene and the samples were assayed for "folic acid" with *S. faecalis* R[4] and for pteroylglutamic acid with *L. casei*.[5] The results are summarized in Table I.

Discussion. Pteroic acid has been found to stimulate the growth of certain lactic acid bacteria under conditions in which a response also occurs to pteroylglutamic acid. In one class of organisms, typified by *L. casei*, a response is produced by pteroylglutamic acid but not by pteroic acid or p-aminobenzoic acid. A second class, typified by *S. faecalis* R, responds to pteroylglutamic acid and pteroic acid, but not to p-aminobenzoic acid. A clue to the function of pteroic acid in this class of organisms may be afforded by the observation[6] that certain enterococci in "resting cell suspensions" convert the "S.L.R. factor," which is pteroic acid with an added formyl group,[7] to pteroylglutamic acid. A third type of organism, exemplified by *L. arabinosus* 17-5[8] responds to either pteroylglutamic acid, pteroic acid, or p-aminobenzoic acid. Studies

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TABLE I.
Assays of Urine Following the Administration of Pteroic Acid.

Amt administered* mg	Route	Assay result†				24-hr urine vol., ml	Apparent excretion of pteroic acid‡		Apparent conversion of pteroic acid to pteroylglutamic acid§	
		S. faecalis R		*L. casei*			% administered dose	Avg %	% admin. dose	Avg %
		Basal mγ/ml	24 hr sample mγ/ml	Basal mγ/ml	24 hr sample mγ/ml					
2	Oral	2	19	11	19	970	2.7			
2	”	2	2	8	6	2330	0	1.4	Insignificant	
5	”	2	18	5	8	1680	1.7			
5	”	2	46	8	12	890	2.4			
5	”	3	32	9	5	1260	2.3	2.1	”	
10	”	2	4	8	8	1770	0.2			
10	”	4	14	10	13	620	0.4	0.3	”	
2	Intravenous	2	150	6	20	2000	46		1.1	
2	”	2	208	5	38	1200	39		1.6	
2	”	2	85	11	36	1170	15	33	1.4	1.4

* The compound was prepared as described in the text. It was found to be 90% pure by the chemical reduction test[3] and by measurement of the extinction coefficient at 365 mμ. When the preparation was assayed in comparison with pteroylglutamic acid values of 32% and .004% of the activity of pteroylglutamic acid were obtained, respectively, with *S. faecalis* R and *L. casei*.

† Calculated in equivalence of pteroylglutamic acid.

‡ Calculated from the assay with *S. faecalis* R, using a factor of 32% (see above) to convert the pteroylglutamic acid equivalence into terms of pteroic acid.

§ Calculated from the assay with *L. casei*, since this organism responds to pteroylglutamic acid and not to pteroic acid. The calculation was adjusted for the purity of the pteroic acid and the molar weight ratios of pteroic acid and pteroylglutamic acid.

of the relative potencies of these 3 compounds for *L. arabinosus*[8] showed that the first 2 are less active on a molar basis than is p-aminobenzoic acid.

In experiments with chicks, pteroic acid[2] and p-aminobenzoic acid[9,10] have been found ineffective as a replacement for pteroylglutamic acid in the diet. Pteroic acid was administered,[11] 5 mg daily for 10 days without effect, to a patient with pernicious anemia in relapse. The patient subsequently responded to treatment with 5 mg of pteroylglutamic acid daily for 6 days.

A study of the urinary excretion of pteroylglutamic acid indicated that about half of the dose, as estimated by assay with *S. faecalis* R, appeared in the urine during 6 hours following the oral administration of 5 mg of the substance. Similar results were obtained when intravenous administration was used.[1] In contrast, pteroic acid when fed by mouth did not appear to be excreted in significant amounts in the urine, but when it was given intravenously a considerable proportion of the administered dose appeared in the urine. This may indicate that the rate of uptake from the gastrointestinal tract is low, or, less probably, that the compound is destroyed in the tract before being absorbed. The results are interesting in view of the low solubility of the monosodium salt of pteroic acid which is in contrast to the moderate solubility of sodium pteroylglutamate. At the pH of the small intestine, the ion formed by the dissociation of only the carboxyl group would be the predominant form of pteroic acid.

A possibility to be considered in the metabolism of pteroic acid is its conversion to pteroylglutamic acid. The respective activities of the 2 compounds for *S. faecalis* R and *L. casei* enable a differential assay to be conducted, especially if pteroic acid is present in amounts considerably in excess of pteroylglutamic acid. In this investigation, the *L. casei* assay value of the urine was very low following the intravenous administration of pteroic acid in contrast to the *S. faecalis* R assay value. This indicated that the urine contained predominantly pteroic acid rather than pteroylglutamic acid. However, a distinct rise in the *L. casei* value was observed, which indicated probable conversion of a small proportion of the pteroic acid to pteroylglutamic acid (Table I).

Summary. 1. Pteroic acid was administered to normal adult human males and the urine was assayed for "folic acid" with *S. faecalis* R and for pteroylglutamic acid with *L. casei*. On the basis of the relative response of these organisms to pteroic acid and pteroylglutamic acid, the apparent concentration of these compounds in the urine was determined.

2. The oral administration of from 2 to 10 mg of pteroic acid resulted in only a very small amount of urinary pteroic acid.

3. Following the intravenous administration of 2 mg of pteroic acid the microbiological assays of the urine indicated that from 15 to 46% of the compound was excreted as pteroic acid and that only about 1% appeared to be converted to pteroylglutamic acid.

4. The excretion studies indicated that only a small proportion of an administered dose of pteroic acid was converted to pteroylglutamic acid and that pteroic acid is poorly absorbed from the gastrointestinal tract.

The assistance of Miss Margaret Regan and Mr. J. W. Boehne, III, is gratefully acknowledged.

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16164

Local Resistance to a Lethal Dose of Formalin.*

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According to Selye[1,2,3] an animal exhibiting an "alarm reaction" in response to a given chemical is able to resist a lethal dose of a chemical of different nature because of a non-specific mechanism of general defense initiated by this "alarm reaction" and called "reaction of adaptation." In repeating certain of Selye's experiments, preliminary to studies on induced immunity to transplantable leukemia, evidence was obtained that questions the general distribution of the cross resistance between the two chemicals employed.

The present communication is concerned with a study of the location of resistance to formalin and adrenalin induced by formol treatment when the sites of pretreatment and lethal doses were varied.

The experimental material consisted of male and female mice of strain C58,[4,5] whose ages varied between 5 and 7 weeks and whose weights varied between 13 and 22 g. These animals received the standard food and care of this laboratory.[6] Solutions of formalin of 4% and 10% (prepared from the commercial solution) and adrenalin in aqueous solution of 1:1000 were used as "alarming" stimuli in subcutaneous injections. The minimum lethal doses of these substances were found to vary according to the site of injection (Table I).

Treatment previous to the lethal dose was administered within a period of 48 hours, using 4 injections. The doses used when all 4 injections were given at the same site were the following: 0.10, 0.20, 0.30, and 0.40 cc of 4% formalin; 0.05, 0.10, 0.12, and 0.15 cc of 10% formalin; or 0.06, 0.08, 0.10, and 0.10 cc of adrenalin; in different experiments the site was the skin of the abdomen, the dorsolumbar region, or the ventral surface of the left hind leg. When the injections were made at different sites in the same mice, they received either 4 doses of 0.15 or 0.20 cc of 4% formalin, or 4 injections of 0.06 cc of adrenalin, the sites being: the extremities, and the abdominal, anterior thoracic, dorsolumbar, and dorsocervical regions. In a few experiments each dose of the treatment with progressive amounts of the chemicals was divided into 4 subdoses, and

TABLE I.

Minimum Lethal Dose of Formalin and Adrenalin Given Subcutaneously in C58 Mice According to the Sites.

Male weight: 18 to 22 g; female weight: 13 to 20 g.

Chemical and concentration		Dorsolumbar	Abdominal	Anterior thoracic	Medial surface hind left leg	Dorsocervical
Formalin	10%	0.20	0.20	0.20	1.15	0.10
Formalin	4%	0.50	0.50	—	—	—
Adrenalin	1%	0.15	0.12	—	—	—

* The author is deeply indebted to Dr. E. C. MacDowell for suggestions during the work and for the valuable critic of this paper.

† Chilean Fellow of John Simon Guggenheim Foundation, year 1944.

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TABLE II.

Number of Mice Surviving Lethal Dose of Formalin or Adrenalin When Injected in the Site of Pretreatment or in a Different Site.

	Pretreatments			Lethal dose		No. of mice	No. surviving after					Total surv.
	Chemical	Dosage	Sites	Chemical	Sites		1st injec.	2nd injec.	3rd injec.	4th injec.	L.D.	
1	Form.	incr.	same	"	—	34	34	34	33	33	—	33/34
2	"	"	diff.	"	—	28	28	18	11	3	—	3/28
3	"	"	same	Form.	same	88	87	85	84	74	65	65/74
4	"	"	"	"	diff.	96*	89	82	77	61	7	7/61
5	"	equal	"	"	same	31	28	28	26	26†	13	13/22
6	"	"	diff.	"	diff.	54	37	35	35	35	9	9/35
7	Adren.	incr.	same	Adren.	"	16	16	15	13	11‡	5	5/ 5
8	Form.	"	"	"	same	55	45	45	41	39§	38	36/38
9	"	"	"	"	diff.	26	26	26	26	26	3	3/26
10	Adren.	"	"	Form.	same	16	16	12	8	6	0	0/ 6
11	"	equal	diff.	"	diff.	10	9	7	4	3	0	0/ 3
12	Untreated controls			"	—	128					11	11/128
13	"			Adren.	—	35					2	2/35

* One group of 21 animals received the treatment in the same site but each dose was distributed in 4 different sites.
†, ‡, § Only 22, 5, and 38 animals, respectively, were injected with the lethal dose.

administered at 4 different sites. The animals were usually subjected to the lethal test dose (the size of the dose varying according to the site) 12 hours after the last of the pretreatments, although in a few cases the lethal dose was administered 2, 3 or 6 days later. When death occurred, it was, in most experiments, at approximately the same time as the death of the controls without pretreatment.

Resistance to the preliminary treatment and lethal dose of formalin depends upon the site of administration (Table II). When progressive doses of this substance were injected into one site, 33 out of 34 animals resisted the 4th pretreatment (line 1), while there were only 3 survivals out of 28 cases when the 4 treatments were given in different sites (line 2). Resistance to the lethal dose also depends on the same factor. When it was administered at the site of pretreatments, 65 out of 74 mice survived (line 3); when the lethal dose was injected at a different site, only 7 out of 61 survived (line 4), a result similar to the controls without pretreatment (survival, 11 out of 128, line 12).

The pretreatment was less effective when sublethal doses of equal amount were used. Animals treated in this manner showed resistance to lethal dose injected at the same site in 13 cases out of 22 (line 5), and the animals which died did so after a longer interval than the controls which had been pretreated at different sites (survival, 9 out of 35, line 6).

Many of the animals treated with adrenalin died during the course of treatment, whether this substance was administered at the same site or at different ones. Some of them, subjected to a lethal dose after 4 preliminary injections, resisted adrenalin even when it was applied at a site different from that of the pre-treatment (survival, 5 out of 5, line 7).

In experiments with cross-resistance, 36 out of 38 mice pretreated with formalin resisted a lethal dose of adrenalin when all injections were at the same site (line 8); when the lethal dose was injected at another site, only 3 out of 26 survived (line 9), with the result that the survival rate approached that

TABLE III.
Anatomical Findings Soon After Treatment with 10% Formalin.
Group I—4 injections of 0.05, 0.10, 0.12, and 0.15 cc.
Group II—4 injections of 0.10, 0.15, 0.20 and 0.25 cc.

Experimental groups	No. of mice	Organ wt (mg)					Local lesion site inj.	General hyperemia
		Liver	Spleen	Thymus	Adrenal	Pancreas		
I	5	726	37	14	5.0*	85	+++	++
		(600-800)	(30-38)	(12-18)	(4.4-5.5)	(68-99)		
II	5	814	46	21	4.5*	95	+++	++
		(700-950)	(38-68)	(10-35)	(4.0-5.2)	(60-155)		
Normal (controls)	5	802	81	47	4.1	141	——	——
		(720-880)	(64-127)	(41-60)	(3.8-4.5)	(90-179)		

* Most of the animals showed adrenal changes: cortex pink and slightly transparent; pale medulla, serous membrane edema doubtful in Groups I and II after treatment. One spontaneous death in Group I and 3 in Group II.

of untreated controls injected with the same dose (2 out of 35, line 13). The animals treated with adrenalin did not show cross-resistance to formalin, whether the lethal dose was injected at the same or at a different site (lines 10 and 11).

Autopsy was made on various animals in order to investigate the anatomical symptoms of the alarm reaction and the reaction of the skin at the site of treatment (Table III). At the termination of the treatment, involution of the thymus was evident. Changes in the suprarenal and pancreas were less conspicuous, with a few exceptions. General hyperemia was present, and digestive hemorrhages were frequently observed in animals treated with adrenalin.

The skin showed changes only when formalin was used, and edema and congestion were apparent during the treatment; several days after the last injection, formation of a necrotic scar became evident.

Histological examination of animals treated with formalin, kindly made by Dr. Richard Miller, revealed the changes of the thymus mentioned under the description of the alarm reaction, increase of mitosis of the cortical cells of the suprarenal, and a necrotic and inflammatory type of skin alteration.

It appears that the induction of resistance to a lethal dose depends upon the nature of the chemical used and upon the site. The animals pretreated with adrenalin evinced resistance to the lethal dose of adrenalin of general character, at least in the one small experiment, but showed no cross-resistance to formalin, either local or general. On the contrary, the animals pretreated with formalin showed only a local resistance, whether they were subjected to a lethal dose of formalin or one of adrenalin. Although no general resistance was observed, the formalin-treatment produced the principal anatomical signs of Selye's "alarm reaction."

These differences observed in mice treated with formalin and adrenalin appear to have their explanation in the differing capacities of these substances to induce local lesions. While the first caused intense manifestations at the site of the injection, culminating in

the formation of a necrotic lesion of the skin, the second, on the other hand, did not produce macroscopically visible local lesions.

Nothing definitive can be said about the possible mechanism of the local resistance observed. It is quite possible that the inflammatory state provoked by the formalin diminished the absorption of the lethal dose.[7,8,9]

Although local resistance is not frequently encountered, it is not a new phenomenon. Rosenthal, Tabor, and Lillie observed that mice surviving tourniquet shock because of salt treatment would survive a repetition of the same shock without salt, if given in the same leg, but would die if it was given in a different leg.[10] Similarly, according to a review by J. Levy, animals in which resistance to arsenic has been built up *per os* do not tolerate a toxic dose administered subcutaneously.[11]

Summary. When mice were pretreated with adrenalin they showed general resistance to a lethal dose of this substance but not cross resistance to formalin, either local or general. On the contrary, animals pretreated with formalin revealed only a local resistance, whether they were subjected to a lethal dose of formalin or of adrenalin. These observations seem to question Selye's interpretation of some of his experiments on general adaptation to the same or different "alarming" stimuli.

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16165

Renal Reabsorption of Methionine in Normal Dogs.*

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It is known that at normal plasma concentrations, reabsorption of amino acids from the glomerular filtrate is practically complete. Experiments in which the plasma concentration has been raised above normal levels have shown, however, that there are differences in the efficiency with which individual amino acids are reabsorbed. These differences are manifested in the rates of reabsorption of the various acids prior to the attainment of the maximal rate (Tm), and also in the levels at which the Tm is reached.[1-5]

The experiments to be described were designed to study the renal reabsorption of methionine. Shortly after they were concluded, experiments utilizing the microbiological method of analysis were reported[5] which indicated that the reabsorption of methionine is practically complete at plasma levels up to 115 mg % (equivalent to about 10.8 mg

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‡ Deceased.

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TABLE I.

The Relationship Between the Amount of *dl* Methionine Alpha-amino Nitrogen Filtered and the Amounts Excreted and Reabsorbed in a Normal Dog.

Dog No. III

Exp. No.		Period	Urine flow, cc/min.	Glomerular filtration rate, cc/min.	Alpha-amino nitrogen					
					Plasma conc. mg %	Urine conc. mg%	Filtered mg/min./ sq.m.	Excreted mg/min./ sq.m.	Reabsorbed mg/min./ sq.m.	Ratio Reabsorbed / Filtered
1		Control	0.37		3.9	3.6		0.03		
	1% methionine, 0.5% inulin	1	4.57	46.5	9.4	2.1	10.1	0.22	9.88	.98
	(Wt, 7.7 kg; S.A.,* 0.437 sq.m.)	2	4.77	46.2	11.4	7.0	12.1	0.77	11.3	.93
		3	6.23	52.2	13.4	11.6	16.0	1.65	14.4	.90
		4	6.37	48.3	15.0	18.1	16.6	2.64	14.0	.84
2		Control	—		3.9	—		—		
	1.3% methionine, 0.5% inulin	1	2.47	49.8	11.0	12.4	12.5	0.70	11.8	.94
	(Wt, 7.7 kg; S.A., 0.437 sq.m.)	2	4.63	51.6	12.9	16.9	15.2	1.79	13.4	.88
		3	5.67	44.8	15.1	22.8	15.5	2.96	12.5	.81
		4	5.60	53.1	16.8	35.3	20.4	4.52	15.9	.78
3		Control	0.17		3.5	6.8		0.03		
	1.5% methionine, 0.5% inulin	1	2.63	61.9	10.4	7.6	14.1	0.44	13.7	.97
	(Wt, 8.2 kg; S.A., 0.455 sq.m.)	2	7.26	65.7	15.5	23.6	22.4	3.77	18.6	.83
		3	7.47	58.9	17.2	30.2	22.3	4.96	17.3	.78
		4	6.67	52.4	18.7	21.0	21.5	3.08	18.4	.86
4		Control	—		—	—		—		
	2% methionine, 0.5% inulin	1	2.57	58.5	11.4	20.7	13.2	1.06	12.1	.92
	(Wt, 9.55 kg; S.A., 0.504 sq.m.)	2	4.63	58.2	14.7	27.7	17.0	2.54	14.5	.85
		3	7.80	57.5	19.6	34.1	22.4	5.28	17.1	.76
		4	8.77	52.2	27.0	42.9	28.0	7.46	20.5	.73

* Surface area in square meters $= 0.112\sqrt[3]{(\text{body wt in kg})^2}$ (Lusk, G., *The Science of Nutrition*, W. B. Saunders Co., pp. 122-123).

% methionine α-amino nitrogen). The results of the present study, based upon α-amino nitrogen determinations and extending the plasma concentrations beyond the levels previously reported, provide further evidence for the high efficiency of the renal reabsorptive process for methionine.

Methods. The experiments were carried out upon 2 healthy female dogs in the manner described in detail elsewhere.[4] Infusions of appropriate concentrations of inulin and dl-methionine in physiological saline were made into the femoral vein at a rate of about 5 cc per minute. Urine collections were made from the bladder with an indwelling catheter and blood samples were taken from the femoral artery by the use of an indwelling spinal needle cut to a length of 4 cm and equipped with a tightly fitting stylet. Periods were 30 minutes in duration. Blood samples of 10 cc were taken at the beginning, mid-point and end of each period and the average amino acid and inulin concentration of the 3 samples employed to indicate the level for the period.

The plasma amino acid concentration was determined manometrically by the ninhydrin-carbon dioxide method of Hamilton and Van Slyke[6] and urinary amino acid was similarly determined by the method of Van Slyke, MacFadyen and Hamilton.[7] Inulin was determined by the method of Hubbard and Loomis[8§] using a Coleman spectrophotometer to measure the intensity of color developed.

Results. Eight different experiments, involving 31 periods, were performed upon one dog and 3 experiments involving 8 periods on another. Plasma levels up to 27 mg % of α-amino nitrogen and filtration rates up to 34.8 mg per minute per square meter of body surface were obtained. In all of the experiments, the efficiency of the renal tubules in reabsorbing methionine from the glomerular filtrate proved to be very high. There was no indication that the maximal rate of reabsorption was reached at any of the levels of glomerular filtration which were attained.

Table I shows the essential data obtained in four of the experiments performed upon Dog III. Each of these experiments was repeated upon the dog with similar results. Inulin clearance has been used as a measure of glomerular filtration rate[9] and the rates of filtration, excretion and reabsorption of α-amino nitrogen have been calculated as previously described.[4] It is seen that as the filtration of amino nitrogen increases through 14 mg/min/square meter reabsorption is well over 90% complete. Beyond this level, the proportion reabsorbed decreased slightly for this dog, but remained remarkably high throughout the experiments. In the experiments upon the second dog (No. IV), plasma α-amino nitrogen concentrations up to 19.8 mg % and filtration rates up to 32.5 mg/min/square meter were attained. Except for the fact that the ratio of reabsorbed : filtered amino nitrogen remained above 0.82, even at the highest filtration levels, the results were in all respects similar to those described for Dog III.

Fig. 1 shows graphically the relationship between the amount of methionine α-amino nitrogen filtered and that reabsorbed in all eight of the experiments performed upon Dog III. It will be noted that although the slope of the curve decreases somewhat beyond filtration rates of around 14 mg/min/square meter, there is no tendency to plateau even at the highest levels attained.

From these experiments it is evident that the tubular capacity for the reabsorption of methionine is relatively high. In terms of α-amino nitrogen it exceeds 25 mg/min/square meter. In this respect, methionine resembles glycine[1] and alanine.[2] These substances manifest reabsorption capacities

[6] Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, 1943, **150**, 231.

[7] Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, 1943, **150**, 251.

[8] Hubbard, R. S., and Loomis, T. A., *J. Biol. Chem.*, 1942, **145**, 641.

§ This method was modified to the slight extent of heating the color-forming solution at 80°C for 14 minutes instead of 8 as prescribed by the authors. This modification assured the maximal development and stability of color.

[9] Smith, H. W., *The Physiology of the Kidney*, Oxford University Press, 1937.

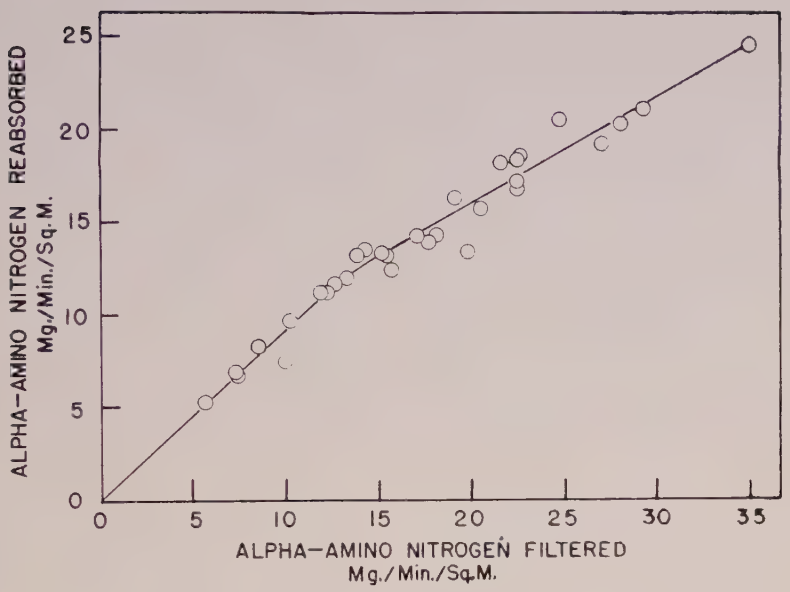

FIG. 1.
The relation between renal filtration and reabsorption of alpha-amino nitrogen of *dl*-methionine in Dog III.

which are considerably higher than the maximal rates reported for leucine, isoleucine and valine[4] and which stand in strong contrast to such low threshold amino acids as arginine and lysine.[3,5]

Summary. The relation between the rate of renal filtration and reabsorption of dl-methionine α-amino nitrogen has been studied in normal dogs. As the amount filtered was increased to 34.8 mg α-amino nitrogen/min/square meter, the highest level attained in these experiments, the rate of reabsorption increased to 24.8 mg/min/square meter and there was no evidence that a Tm value was obtained. The data demonstrate the high efficiency of the renal mechanism for the reabsorption of methionine.

16166 P

Cataracts Resulting from a Deficiency of Phenylalanine in the Rat.*

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Experimental cataracts in the rat have been shown to result from deficiencies of either of 2 of the essential amino acids. What apparently were cataracts due to tryptophane deficiency in the rat were first described by Curtis, Hauge and Kraybill.[1] Totter and Day[2] later made a careful study of the cataracts resulting from this deficiency. Recently,

* This study was aided by grants from the John and Mary R. Markle Foundation and from the Division of Grants, National Institute of Health, U. S. Public Health Service.

1 Curtis, P. B., Hauge, S. M., and Kraybill, H. R., *J. Nutrition,* 1932, **5**, 503.

2 Totter, J. R., and Day, P. L., *J. Nutrition,* 1942, **24**, 159.

Sydenstricker, Schmidt and Hall[3] have reported the development of cataracts in rats on a histidine-deficient diet. The study reported here was occasioned by the discovery of a well-developed cataract in a rat fed on a diet deficient in phenylalanine.

Methods. The rats used were from a Wistar strain. When 25-28 days of age they were placed in individual cages and given experimental diet and water *ad libitum*. The phenylalanine-deficient diet and a similar control diet used were those described by Sydenstricker, Hall, Bowles and Schmidt.[4] The development of cataracts was studied in 15 rats from 5 litters which were fed the phenylalanine-deficient diet. Seven rats from the same litters were fed the control diet. Three times weekly the eyes were examined for lenticular abnormalities with the biomicroscope after dilating the pupil with 0.5% solution of atropine sulphate in physiological saline solution. As soon as possible after the death of each deficient rat the lenses were removed, immersed in saline, and examined under a dissecting microscope.

Three deficient rats with cataracts of varying degrees of development were changed to the control diet so that any resulting regression of the lenticular changes could be observed.

Results. Definite lenticular changes were observed in all but 2 of the 15 rats in from 17 to 33 days (mean, 22 days). These changes continued to develop, resulting in a progressive lessening of the transparency of the lenses until at the time of death, after 20-53 days on the diet, various degrees of opacity were observed. The earliest change was a slight haziness of the lens substance. Next the lens star became visible. This was followed by a separation of the superficial fibers, and sometimes by a rough granular appearance of the epithelial layer. No changes in the lens capsule were observed. Finally, in the posterior portion of the lens a dense central opacity developed, eventually filling the central portion of the lens and leaving only a narrow peripheral portion comparatively clear.

Changing the deficient rats to the control diet resulted in a reversal of only the more superficial changes in the lens.

In none of the control rats were any lenticular changes observed.

Summary. The lenticular changes observed to result from phenylalanine-deficiency in the rat were diffuse haziness, opacity of the star, progressive separation of the fibers, granular changes in the epithelial layer and the development of dense central cataracts. Only the more superficial changes in the lenses were found to be reversed by adding *dl*-phenylalanine to the diet.

[3] Sydenstricker, V. P., Schmidt, H. L., Jr., and Hall, W. K., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 59.

[4] Sydenstricker, V. P., Hall, W. K., Bowles, L. L., and Schmidt, H. L., Jr., *J. Nutrition*, 1947, **34**, 481.

16167

Effect of Podophyllin on Tumor Cells in Tissue Culture.*

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Podophyllin, N.F. is a mixture of substances derived from the roots and rhizomes of the mandrake plant by hot ethanol extraction and subsequent precipitation in dilute HCl. Various preparations of the mandrake plant have been in use for at least a hundred years as laxatives and more recently as supposed

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liver stimulants.

The constituent chiefly responsible for the drastic laxative action of podophyllin is podophyllotoxin.[1] This is a highly toxic compound which causes mucosal inflammation and gastroenteritis. It likewise involves the nervous system resulting (in the cat) in disturbance of coordination of the posterior extremities, rapidly increasing weakness, increased respiratory rate, violent colonic cramps and death in coma.[2,3] The parenteral administration of podophyllin to chickens likewise resulted in nervous system involvement and cytological examination revealed damage or complete degeneration of the cerebellar Purkinje cells and other nervous elements.[4]

Kaplan[5] demonstrated that a suspension of podophyllin in oil was highly effective against *Condylomata acuminata*. These results were confirmed and extended by King and Sullivan[6,7] and by Sullivan and Blanchard.[8] These investigators likewise noted the similarity of podophyllin to colchicine in its effects on venereal warts. Sullivan and Wechsler[9] have shown that saturated aqueous solutions of podophyllin block mitosis in the root tips of *Allium cepa*. In very dilute solutions both podophyllin and podophyllotoxin destroy the mitotic spindles of cleaving *Asterias* and *Arbacia* eggs, as discovered recently by Cornman.[10]

The effects of podophyllin on *Condylomata acuminata* and *Allium* made this compound of interest to us as a possible therapeutic agent in the treatment of cancer. Our interest was increased by the incidental use in tissue culture of placental serum from a patient previously treated with podophyllin for *Condylomata acuminata*. This placental serum caused severe damage to mouse tumor cells growing in roller tubes without affecting normal cells growing in the same tubes.

Material and Methods. It was considered desirable to design a method for the detection of materials of possible value in the chemotherapy of cancer which take advantage of the tissue culture techniques for mammalian cells. Since we planned to test a large number of materials it was necessary that the test be one which gave a maximum of information within a short time. The roller tube technique of Gey and Gey[11] was consequently adopted. In this procedure thin-walled pyrex glass tubes (150 mm x 15 mm) are used. Each tube contains 6 pieces of normal embryonic mouse skin arranged in a row along the bottom third of the tube and a row of 6 mouse tumor fragments similarly arranged on the opposite wall of the tube. Occasionally a third row of 6 pieces of a second tumor is placed in the tube. The mouse tumors used have been in-strain transmittable mouse sarcoma L946 from C-57 mice and lung tumor MA387 from AK mice.

The tissue fragments are held in place with a thin layer of chicken plasma clot. The nutrient medium totaling one ml consists of 0.4 ml Gey's solution, 0.2 ml chick embryo extract, 0.1 ml human placental serum and 0.3 ml horse serum. The tubes are closed with sterile rubber stoppers and incubated at 37°C in a rotating drum.

Our customary procedure is to incubate the tubes for 24 hours, then examine and grade the cells that have grown or migrated from each explant. Nutrient medium to the volume of 0.1 ml is withdrawn from the tube and the test material in a volume of 0.1 ml is then added to the nutrient medium. The tubes are incubated for another 24 hours, and the growing cells again graded.

1 Magnus, R., *Handbuch der Experimentellen Pharmacologie*, ed. A. Heffter, vol. 2, pt. 2, p. 1645, J. Springer, Berlin, 1924.

2 Viehover, A., and Mack, H., *J. Am. Pharm. Assn.*, 1938, **27**, 632.

3 Chenoweth, M. B., Hunt, C. C., and Philips, F. S., 1947, personal communication.

4 MacCardle, R. C., and Perrault, A., *Fourth Int. Cancer Cong.*, 1947, abstract.

5 Kaplan, I. W., *New Orleans Med. and Surg. J.*, 1942, **94**, 388.

6 King, L. S., and Sullivan, M., *Science*, 1946, **104**, 244.

7 Sullivan, M., and King, L. S., *Arch. Derm. Syph.*, 1947, **56**, 30.

8 Sullivan, M., and Blanchard, D., *Bull. Johns Hopkins Hosp.*, 1947, **81**, 65.

9 Sullivan, B. J., and Wechsler, H. I., *Science*, 1947, **105**, 433.

10 Corman, I., *Biol. Bull.*, 1947, in press.

11 Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, **27**, 45.

The tumor tissue is removed at this time and implanted into susceptible mice. The normal tissue is grown for 4 days longer in fresh normal nutrient medium and again graded.

In grading the outgrowths of cells from the original tissue fragments at 24 hours, 48 hours, and again at 6 days, all visible microscopic changes that can be adequately observed in living cells are examined. The following points are noted and a grade of 0 to 4 assigned to all except "growth" for which the score runs from 0 to 6; 1) "growth," 2) inhibition of lysis of the plasma clot, 3) number of abnormally rounded cells, 4) severity of cell rounding, 5) number of cells with granulated cytoplasm, 6) intensity of cytoplasmic granulation, and 7) extent of cell disintegration.

The scores for 3, 4, 5, 6, and 7 are added and by subtracting similarly calculated control values, a damage score for tumor cells termed the "Selective Index" is derived. This is represented by the formula—

$$SI = \left[(T^{E}_{48} - T^{E}_{24}) - (N^{E}_{48} - N^{E}_{24}) \right] - \left[(T^{C}_{48} - T^{C}_{24}) - (N^{C}_{48} - N^{C}_{24}) \right]$$

where T represents tumor tissue, N represents normal embryonic mouse skin, the superscript E represents experimental tube, the superscript C represents control tube and the subscripts 48 and 24 refer to the composite damage grades at those hours.

The Selective Index plus the scores for "growth" and inhibition of lysis of the plasma clot are useful in assessing the effects of chemotherapeutic candidates. The comparative effects on normal and tumor tissue of a large series of materials can be easily compared on this basis. The term "growth" here means both cell migration and cell proliferation and actually represents simply the increase around the original explant in the area which is covered by cells originating in the explant.

Selective Indices of 0 to 10 are considered negative, 10 to 30 are considered significant and over 30 highly significant. Less than 10% of all materials thus far tested in this manner have given significant differential effects. A number of substances that have been used more or less extensively in the treatment of human cancer have given negative results in this test. These include various nitrogen mustards, heptaldehyde, urethane, Fowler's solution, benzene and stilbamidine.

The podophyllin in these experiments was precipitated from a 95% ethanol solution in 10% gum acacia and subsequently diluted in 0.87% NaCl to the desired concentrations.

Experimental. Table I shows the results of a representative experiment with podophyllin. In this experiment each tube contains 6 pieces each of normal fetal skin, L946 and MA387. The figures presented each represent the sum of the scores for 6 tissue fragments. In this experiment the terms $(T^{48}_{C} - T^{24}_{C})$ and $(N^{48}_{C} - N^{24}_{C})$ were zero in parallel control preparations and $(N^{48}_{E} - N^{24}_{E})$ can be seen to be zero from Table I. The Selective Index was calculated therefore simply by subtracting the 24-hour tumor damage scores from the 48-hour tumor damage scores and averaging the results from the 2 tubes.

It is seen that podophyllin is significantly more damaging *in vitro* to mouse tumors L946 and MA387 than to normal embryonic mouse tissue including fibroblastic and epithelial elements.

Under the influence of podophyllin the tumor cells, which are typically spindle shaped and have long slender cytoplasmic processes, become rounded or lobose in form. Migration of the cells from the explant appears to be repressed. Normal fibroblast cells show the same changes but only at much higher concentrations of podophyllin. The tumor cells (unstained) which normally spread out in a loose interlacing sheet, coalesce into a dense and closely packed sheet or ring around the original explant. Isolated cells in the plasma clot appear rounded and smaller, as though some dehydration had occurred. The nucleus disappears from sight leaving only granular cytoplasm. There is a striking change in the normally fine cytoplasmic granulation. The fine granules are replaced by larger granules which aggregate in clumps throughout the cytoplasm. The non-granular portion of the cytoplasm loses its fine structure and assumes a clear hyaline appearance. Although few

TABLE I.
Screening Test—Podophyllin, 12 mg/1; N, Normal Fetal Mouse Skin; L, Sarcoma, L946; M, Lung Tumor MA387.

	Tube No. 1 24-hr data			Tube No. 2		
	N	L	M	N	L	M
Growth	12	22	16	18	29	15
Lysis inhibition	24	21	20	24	22	17
% rounding	0	1	0	0	1	0
Degree rounding	0	3	0	0	3	0
% granulation	24	24	24	24	24	24
Degree granulation	12	12	12	12	12	12
Disintegration	0	0	0	0	0	0
Total	36	40	36	36	40	36
	48 hr data					
Growth	14	25	19	18	24	19
Lysis inhibition	24	14	19	24	18	16
% rounding	0	24	20	0	24	20
Degree rounding	0	12	9	0	18	11
% granulation	24	24	24	24	24	24
Degree granulation	12	18	17	12	18	18
Disintegration	0	0	1	0	0	0
Total	36	78	71	36	84	73

Selective index of L946 = 41.
Selective index of MA387 = 36.

details of nuclear structure can be seen in the living cells, stained preparations indicate considerable nuclear derangement. The chromatin is represented by disorganized fragments in most cases and considerable degree of pycnosis in others. A slight amount of cellular disintegration often is manifest by the presence of cellular debris in the culture. Essentially the same phenomena occur in normal cells but only at higher concentrations of podophyllin.

Selective damage to the tumor cells as compared with normal cells is obtained with podophyllin over a concentration range of 0.08-20.0 mg/1. Within these concentrations the Selective Index varies from 10 to 45, with the highest scores occurring in the concentration range of 0.3-2.5 mg/1.

This picture of cellular damage and destruction is reversible under some conditions. When podophyllin concentrations of 5 mg/1 or less are employed, and the podophyllin removed after 24 hours' treatment by replacement with fresh normal medium, the normal cells may recover normal appearance and resume normal growth. Tumor cells, on the other hand, have not shown recovery unless the concentrations employed were 0.6 mg/1 or below.

This compares with results found with crude penicillin, from which normal fibroblasts quickly recovered whereas sarcoma cells died.[12]

The specific effects of podophyllin and its components on karyokinesis and cell division are of considerable interest, particularly in view of the fact that nuclear damage including abnormal mitosis has been produced in sarcoma L946 and lung tumor MA387 growing in mice. In these experiments extensive damage to mouse tumors *in vivo* was produced by the parenteral administration of podophyllin. Studies on cell division as well as investigations of the biochemical and carcinoclastic actions of this material are now in progress.

Tissue culture tests with podophyllotoxin indicate that this compound is not as effective as crude podophyllin in causing selective damage to tumor cells. Also, the concentration range within which these slight effects are obtained is quite narrow, in contrast to the wide effective concentration range of podo-

[12] Cornman, I., *J. Gen. Physiol.*, 1944, **28**, 113.

phyllin.

Conclusions. 1. Podophyllin exerts a selective damaging effect on mouse tumor cells in tissue culture over the concentration range 0.08-20.0 mg/l. 2. This damaging effect is more easily reversible in normal than in tumor cells. 3. Podophyllotoxin is not as effective as podophyllin in causing selective tumor damage. 4. *In vivo* studies with tumor-bearing mice confirm the selective tumor damaging effects of podophyllin which were first noted in tissue culture preparations.

16168

Experimental Alteration of the Ability of Tumor Cells to Lyse Plasma Clots *in vitro*.*

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One of the outstanding characteristics of tumor cells when grown in tissue culture is their ability to lyse the plasma clot in which they are embedded. This liquefaction of the clot has been mentioned by many investigators. Drew,[1] comparing cultures of normal and malignant tissues *in vitro,* noted that a 24-hour culture of mouse embryo heart tissue showed a ring of growth around the periphery of the original fragment, while a corresponding culture of mouse sarcoma showed a similar ring of growth separated from the original explant by a circular area of liquefaction. The growth of mouse sarcoma in rat plasma was described by Lambert and Hanes[2] as being "ring-form," since lysis of the clot allowed the contracting fibrin to retract from the original fragment, leaving the fragment situated on the periphery of the circle of cells like the setting in a signet ring. Carrel and Burrows[3] attributed their lack of success in the cultivation of human carcinoma to rapid liquefaction of the plasma clot.

It is important in the analysis of the nature of this lytic process to note that lysis of a plasma clot can be induced by normal tissue. Over 30 years ago, Fleisher and Loeb[4] examined the fibrinolytic effect of various tissue fragments surviving *in vitro* on the plasma of different animals. In general, it was found that mammalian tissues lysed clots formed from mammalian plasma, but had no lytic effect on chicken plasma clots. It was further reported that normal chicken tissues were completely ineffective in producing lysis of either mammalian or chicken plasma clots. The liquefying power of tissues usually decreased when a large amount of chicken plasma was added to the standard rabbit plasma clot.

Very recently, Astrup and Permin[5] reported that tissue slices from different mammals (ox, pig, rabbit and rat) produced lysis of the ox fibrin clots in which they were embedded, the degree of lysis depending on the organ and species of animal used. They, too, found that chicken tissues did not cause lysis of such clots. They suggested that a profibrinolysin in the plasma is activated by a cellular kinase, and thereupon causes lique-

* This research has been supported by grants from the Elsa U. Pardee Foundation and the Whiting Foundation.

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1 Drew, A. H., *Brit. J. Exp. Path.*, 1922, **3**, 20.

2 Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, **13**, 495.

3 Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, **13**, 571.

4 Fleisher, M. S., and Loeb, L., *J. Biol. Chem.*, 1915, **21**, 477.

5 Astrup, T., and Permin, P. M., *Nature*, 1947, **159**, 681.

TABLE I.

The Effect of Varying the Aqueous Humor/Chicken Serum Ratio in the Supernatant of Roller-Tube Cultures of Sarcoma 180. Data represent average of 2 or more experiments. A.H. = Aqueous Humor; C.P. = Chicken Plasma; C.S. = Chicken Serum; 37°C.

Clot	Supernatant concentration of AH to C.S.	Lysis		Growth	
		24 hr	72 hr	24 hr	72 hr
AH/CP = 1:2	3:1	0	2.0	2.5	4.5
,,	2:1	0	1.0	2.0	4.5
,,	1:1	0	0	3.0	4.5
,,	1:2	0	0	1.5	4.0
,,	1:3	0	0	2.5	4.5

TABLE II.

Growth of Sarcoma 180 in the Absence of Serum. Data from one experiment but are representative of additional duplicate trials. 37°C.

Clot	Supernatant (drops)		Lysis 48 hr	Growth 48 hr
	Salt solution	Chicken embryo extract		
Chicken plasma	10	0	0	3.5
,, ,,	9	1	0	4.5
,, ,,	8	2	0	4.0
,, ,,	7	3	0	4.5
,, ,,	6	4	0	4.5

faction although they have been unsuccessful in extracting such a component from tissue.

In our experiments, mouse Sarcoma 180 was grown in roller tubes in a chicken plasma clot with a supernatant ordinarily composed of a balanced salt solution,[6] mammalian serum and chicken embryo extract (one part minced whole embryo to one part balanced salt solution). In such a preparation the clot was partly liquefied within 24-48 hours. This liquefaction sometimes continued until the tissue fragment was entirely surrounded by a ring of liquefaction. Our investigation was concerned with attempts to control such liquefaction and to examine the nature of the mechanism involved by altering the medium in which the tumor is customarily grown.

Material and Methods. The ability of various tumors to grow in the anterior chamber of the rabbit's eye suggested the use of aqueous humor in tissue culture. In our first experiments, the aqueous humors of rabbit, sheep or steer were incorporated in the plasma clot. The effect of modifying the serum content of the supernatant nutrient medium was also examined. Preliminary experiments were carried out in Maximow slides in which hanging drop cultures were set up with various ratios of aqueous humor and chicken plasma in the clot. Subsequent experiments were carried out in roller tube cultures following the technique of Gey and Gey.[6] Tissue fragments were oriented in glass culture tubes and then covered with a layer of chicken plasma. After clotting of the plasma was complete, the supernatant fluid was added and the tubes were incubated at 37°C in a rotor. Seven to 12-day-old mouse Sarcoma 180 tumors, carried in CFW mice, were used in all the experiments. The degree of lysis was graded by dividing the perimeter of the tumor fragment into four quadrants, each with a value of one. Thus, lysis in one quadrant was assigned a grade of 1; 2 quadrants a grade of 2, etc. The amount of growth was graded in a similar manner. A grade of 4 indicated a complete fringe of growth around the fragment. When the width of growth was equal to the diameter of the fragment, a grade of 5 was assigned.

Results. In Maximow slide hanging drop preparations in which aqueous humor was incorporated in chicken plasma clots no lysis of the clot appeared over a 4-day period, al-

[6] Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, **27**, 45.

though there was extensive tissue growth. The most vigorous growth was obtained with concentrations of aqueous humor to chicken plasma of 1:1 and 1:2. Normal growth of the tissue for a period exceeding 4 days has not been successful, the cells becoming extremely granular and rounded, and eventually disintegrating.

All subsequent experiments were carried out in roller tubes. In these preparations lysis of the aqueous humor/chicken plasma clots occurred when a supernatant fluid containing mammalian serum was present. By varying the constituents of the supernatant, it was possible to block this lytic action. If the ratio of aqueous humor to chicken serum was 1:1 or 1:2 in the supernatant fluid, no lysis occurred. Further experiments (Table I) showed that the chicken serum was the responsible factor in preventing lysis. Aqueous humor did not inhibit lysis. On the contrary, if present in high enough concentration, it promoted fibrinolysis.

Table I shows that as the concentration of aqueous humor decreased and the concentration of chicken serum increased, the amount of lysis decreased. At an aqueous humor/chicken serum ratio of 1:1 in the supernatant, lysis was completely absent and growth was apparently unaffected. With higher concentrations of chicken serum in the supernatant, lysis was similarly absent although growth appeared to be adversely affected.

When all sera were omitted from the supernatant fluid, leaving only the balanced salt solution and chicken embryo extract, no lysis of the chicken plasma clot occurred, although there was good growth. The addition of chicken embryo extract did not stimulate lytic activity even though more extensive growth occurred. These data are presented in Table II.

Table III summarizes the roller tube experiments in which the supernatant medium was altered in attempts to prevent lysis of the clot. In these experiments a clot composed solely of chicken plasma was employed.

It will be noted that the presence of avian serum in the supernatants is correlated with the occurrence of little or no lysis, while the presence of mammalian sera resulted in a significant degree of lysis. The addition of sufficient amounts of chicken serum (replacing an equal volume of the balanced salt solution) to the mammalian sera resulted in loss of lytic activity, rabbit serum being an exception—at least at the concentrations tested.

TABLE III.

Plasma Clot Lysis at 48 Hours in Roller Tube Cultures of Mouse Sarcoma 180 Cells in the Presence of Various Sera. 37°C. C.P. = Chicken plasma; H.S. = Horse serum; C.S. = Chicken serum; R.S. = Rabbit serum; Hu.S. = Human serum.

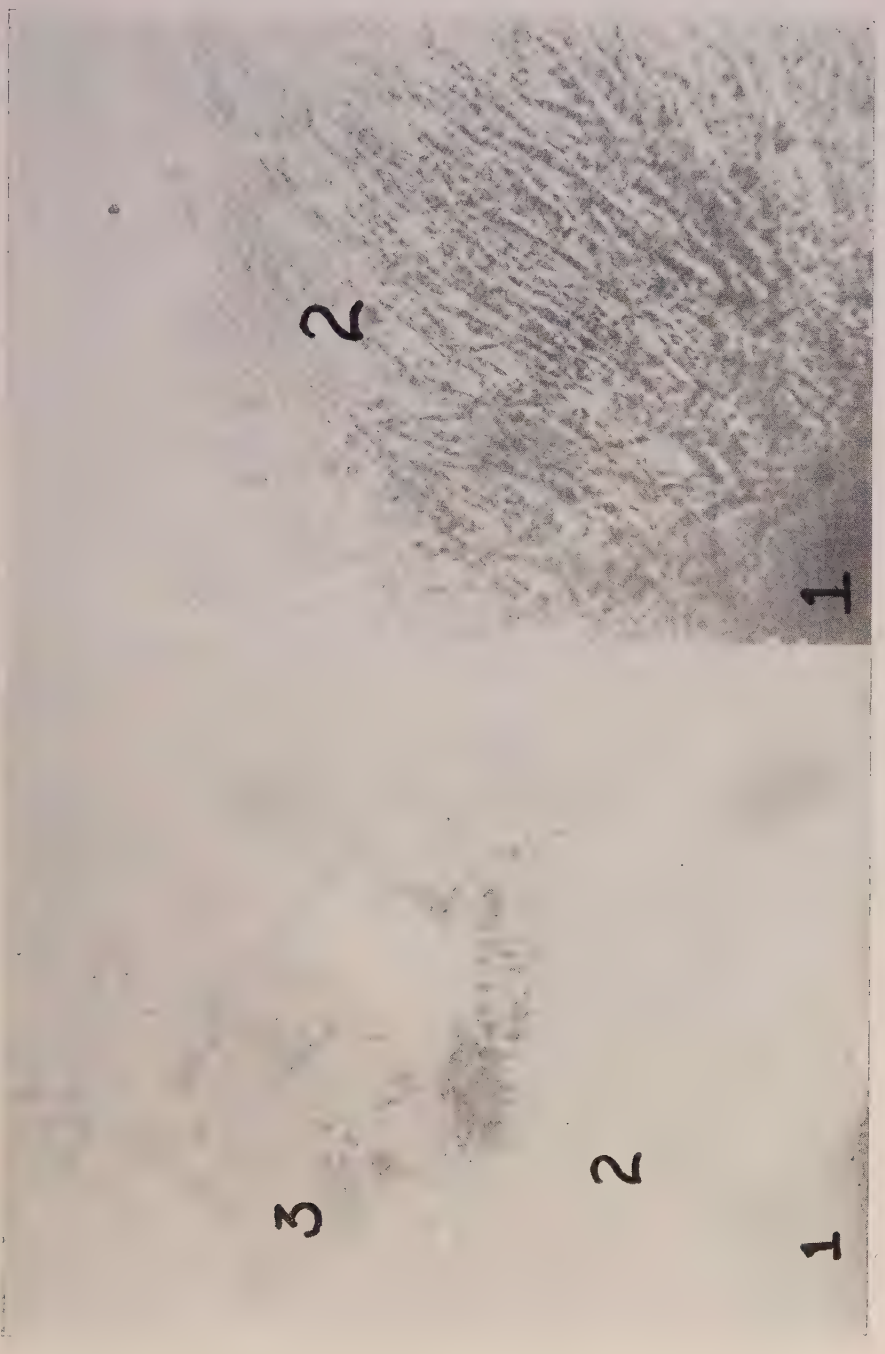
Clot	Supernatant*	Lysis	Growth
C.P.	Chicken serum	0	4.5
,,	Duck serum	0	4.5
,,	Horse serum	2.0	5.0
,,	H.S. + C.S. (2:3)	0	5.0
,,	Rabbit serum	2.0	4.5
,,	R.S. + C.S. (2:3)	2.0	5.0
,,	Human serum	4.0	4.5
,,	Hu.S. + C.S. (2:3)	2.5	4.5
	Hu.S. + C.S. (2:4)	1.0	4.5

* Includes a balanced salt solution and chicken embryo extract.

With human serum in the supernatant, the liquefaction at 72 hours was so complete around the fragment that it was occasionally washed away, leaving an empty space surrounded by a ring of cells. The use of serum previously heated at 56°C for 3 hours resulted in the complete absence of lysis and rapid growth of the cultures. Fig. 1 shows the effect of heating the serum.

Discussion. Inasmuch as omitting serum, using heterologous serum, or heating the serum affects the amount of liquefaction, it seems probable that serum contains a factor essential to the lytic mechanism. Since the area of liquefaction is invariably contiguous with the tumor fragment, it appears that the tissue also contributes to the lytic mechanism. The experimental data presented are in harmony with blood enzyme studies[7] in which it has been suggested that an active proteolytic enzyme is produced as the result of an interaction of a cell activator (streptokinase) and the profibrinolysin normally present in plasma and serum. According to this theory heat in-

[7] Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.



A B

FIG. 1.
72-hour growth of Sarcoma 180 using unheated and heated human serum in the supernatant. Living cultures in roller tubes, × 80.
A—unheated human serum; 1—original explant; 2—lysed area; 3—moribund cells.
B—heated human serum; 1—original explant; 2—extensive outgrowth of Sarcoma 180 cells.

activation of the serum containing profibrinolysin or the omission of serum from the culture medium explains the lack of lytic activity in such cases. The different degrees of lysis occurring when various sera are used may be due to differences in the amount of profibrinolysin present in the different sera. Correspondingly, mammalian aqueous humor can be considered as a dilute mammalian serum which contains only profibrinolysin.

When mammalian Sarcoma 180 is grown in the presence of avian serum, the absence of lysis in this case may be due to (1) lack of sufficient profibrinolysin in the serum, or (2) the inability of mammalian tissue to activate avian profibrinolysin. Since the Rous chicken sarcoma causes extensive liquefaction of chicken plasma clots,[8] it appears that chicken plasma does contain profibrinolysin. It is more likely, therefore, that mammalian tissue is unable to activate avian profibrinolysin. The inhibitory effect of chicken serum on the lytic activity of various mammalian sera may be explained by assuming that the profibrinolysin of the chicken serum combines with the activator from the mammalian tumor Sarcoma 180 to form an inactive complex:

Kinase (Mammalian tissue) + Profibrinolysin (Mammalian serum) ⟶ Fibrinolysin (Active)

Kinase (Mammalian tissue) + Profibrinolysin (Avian serum) ⟶ Kinase-Profibrinolysin (Inactive)

Thus, if sufficient avian serum is added to mammalian serum in the presence of mammalian cells, it effectively removes the mammalian kinase through formation of the inactive complex and prevents activation of the mammalian profibrinolysin and subsequent lysis of the fibrin.

This hypothesis is further supported by the data presented in Table IV. Chicken serum previously heated to 56°C for 3 hours was added to a mammalian tissue preparation with a supernatant containing mammalian serum. No inhibition of the lytic process was observed.

The absence of lysis in preparations in which heated human serum was used exclusively in the supernatant might be explained by postulating the activation of inhibitory substances due to prolonged heating at 56°C. However, the addition of heated human serum to supernatants containing only normal human serum did not result in any significant effect on the lytic process. This indicates that the heated human serum did not contain an inhibitor, and that the absence of lysis in tubes in which the supernatant contained only heated serum is best explained on the basis of destruction of a proenzyme or enzyme concerned with fibrinolysis.

TABLE IV.

Effect of Heated Serum on Lysis in the Presence of Unheated Human Serum. Each datum represents duplicate determinations. C.P. = Chicken plasma; C.S. = Chicken serum; Hu.S. = Human serum. Roller tube cultures, 37°C.

Clot	Supernatant*	48 hr Lysis	48 hr Growth
C.P.	Hu.S. + C.S. (2:4)	1.0	4.5
,,	,, + heated C.S. (2:4)	3.5	4.5
,,	,, + (2)	3.5	4.0
,,	,, + (6)	3.5	4.0
,,	,, + heated Hu.S. (2:2)	4.0	4.0
,,	,, + ,, ,, (2:4)	3.0	4.0

* Includes a balanced salt solution and chicken embryo extract.

Fischer,[8] working with the Rous chicken sarcoma, which liquefies chicken plasma clot, was able to grow this tumor without liquefaction in a clot made from rabbit plasma. In this case it may be that the reciprocal event occurs: avian tissue kinase forms an inactive complex with mammalian profibrinolysin. Fischer further found that on the addition of fresh chicken serum to the rabbit plasma, lysis of the clot occurred; whereas chicken serum heated at 56°C for 3-4 hours produced no such action. Since chicken tumor cells caused liquefaction of the clot in the presence of chicken plasma or chicken serum but not in the presence of rabbit plasma, Fischer concluded that lysis occurred only in the presence of homologous plasma or serum. It is not clear how exclusive his use of the term "homologous" is intended to be. We have shown that differences in sera from other mammalian orders are not sufficient to prevent lysis by mouse Sarcoma 180. Lambert and Hanes[9] demonstrated that when rat and mouse sarcoma cells were grown in the plasma of various animals, liquefaction occurred in the presence of the plasma of guinea pig, rabbit, dog and human (the latter producing the greatest amount of liquefaction). In goat plasma there was neither growth nor lysis. Although there was good growth in pigeon plasma, the authors imply

8 Fisher, A., *Nature*, 1946, **157**, 442.

9 Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, **14**, 129.

that there was no lysis.

The anti-lytic effect of chicken plasma used in culturing mammalian tumors has been established by other investigators. Gey and Gey[6] have reported that the use of a large amount of chicken plasma helped to prevent early liquefaction, but sometimes delayed growth. A strain of sarcoma cells derived from a dibenzanthracene mouse tumor was cultured *in vitro* for more than two years by Jacoby,[10] with almost no liquefaction of the chicken plasma clot. The supernatant consisted of chicken serum, chicken embryo juice and Tyrode solution. Lewis and Strong,[11] in a survey of over 50 different spontaneous mouse tumors in tissue culture, found that the cultures rapidly liquefied the clot when it was composed of mouse plasma or a mixture of mouse and chicken plasma. In chicken plasma, however, such liquefaction did not occur.

In assigning the profibrinolysis to the serum and the kinase to the cells we are following the current ideas of Christensen and McLeod.[7,12] Our data are not such as to eliminate the inverse possibility: profibrinolysin in the tissue activated by a blood kinase. We cannot assume that there are differences only in the amount of profibrinolysin of various animal sera. There may be differences in the amounts of activator released by the cells of animals of different orders, since Fleisher and Loeb,[4] and Astrup and Permin[5] have reported differences in the lytic activity of tissues from various animals.

Summary. 1. Mouse Sarcoma 180 cells, in the presence of mammalian serum, cause lysis of chicken plasma clots. 2. Mammalian aqueous humor has slight influence on the fibrinolytic process. 3. When Sarcoma 180 is grown in the presence of avian serum, no lysis occurs. If a sufficient quantity of chicken serum is added to a supernatant fluid containing mammalian serum there is a decrease in the amount of clot lysis. 4. Lysis of the plasma clot by Sarcoma 180 does not occur when mammalian serum previously heated to 56°C for 3 hours is used, or when all serum is omitted from the supernatant. 5. The different degrees of lysis obtained when sera from various mammalian orders are used may be due to differences in the amount of profibrinolysin contained in the various sera. 6. There are two factors necessary for the lytic mechanism, tentatively classified as a profibrinolysin from the serum, and an activator of the profibrinolysin derived from the tissue.

[10] Jacoby, F., *Nature*, 1943, **152**, 299.

[11] Lewis, M. R., and Strong, L. C., *Am. J. Cancer*, 1934, **20**, 72.

[12] Christensen, L. R., and McLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

16169

Dichotomy Between Hypophyseal Content and Amount of Circulating Gonadotrophins During Starvation.*

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The amounts of gonadotrophin contained within the pituitary gland have often been assumed to parallel the amount produced and released into the blood stream. Since the gonadotrophic hormone content of the hypophysis must be the resultant of the amounts produced and the amounts released, they do not necessarily parallel each other. It is the purpose of this investigation to assess the effect of acute starvation on pituitary gonadotrophic hormone content and its release into the circulation.

The effective amount of circulating gonad-

* Supported in part by a grant from the Eli Lilly Company through the courtesy of Dr. D. C. Hines.

otrophins appears to drop markedly during starvation as judged by ovarian atrophy, uterine atrophy, and anestrus vaginal smears in rodents,[1–7] and amenorrhea in women. The target organ atrophy is not due to a refractory state of the ovaries but to absence of gonadotrophins. This is suggested by the capacity of the ovaries of starved rodents to respond to injections of gonadotrophins[1,3,4,6,7] and the reduced urinary gonadotrophin output in women.[8,9]

Hypophyseal content of gonadotrophins has been determined in chronically starved rats and found to be decreased by Mason and Wolfe[2] and Werner[4] and not changed from normal by Marrian and Parkes.[1] In more acutely starved rats Pomerantz and Mulinos[10] could detect no decrease from normal. Each group of investigators used the method of implanting entire pituitary glands into the recipient assay rats. Since, following implantation, necrosis, growth and elaboration, or encapsulation may occur, the delivery of gonadotrophic hormone is exceedingly variable. This alone may account for the divergent results reported.

Methods. Adult (5 months old) Sprague-Dawley female rats were divided into three groups: Control rats fed *ad libitum;* rats starved for 12 days; and rats starved for 12 days and injected with a suspension of rat

TABLE I.

Procedure	Donor rats: No. rats	Body weight: Before g	Body weight: After g	Body weight: % change	Ant. Pituit. wt, mg	Uterine wt, mg	Ovarian wt, mg	Vaginal smears	Recipient assay rats: Uterine wt, mg: with fluid	Uterine wt, mg: without fluid	Ovary wt, mg	No. rats
Controls fed *ad lib.*	7	219	229	+ 5	9.7	501	63.0	cycling	175	110	21.3	7
Starved 12 days	5	223	142	- 36	6.1	195	30.3	atrophic in 4 days (2-6 days)	321	152	28.2	5
Starved 12 days, RAP* days 9 to 11	6	222	137	−38	9.6	368	73.0	cornified following RAP inj.	302	145	20.2	6
								Uninj. assay rats	41	41	15.6	9
Controls fed *ad lib.*	5	257	261	+ 2	12.5	646	67.1	—	225	130	13.0	5
Starved 16 days	7	253	156	−38	8.6	202	26.1	—	248	124	14.5	7
								Uninj. assay rats	42	42	13.5	10

* Rat anterior pituitary gland suspensions, derived from rats castrated 10 months previously, were injected twice daily for 3 days, a total of ½ gland per rat.

[1] Marrian, G. F., and Parkes, A. S., *Proc. Roy. Soc. London,* 1929, **105**b, 248.

[2] Mason, K. E., and Wolfe, J. M., *Anat. Rec.,* 1930, **45**, 232.

[3] Mulinos, M. G., Pomerantz, L., Smelser, J., and Kurzrok, R., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 79.

[4] Werner, S. C., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 101.

[5] Mulinos, M. G., and Pomerantz, L., *J. Nutrition,* 1940, **19**, 493.

[6] Stephens, D. J., and Allen, W. M., *Endocrinology,* 1941, **28**, 580.

[7] Drill, V. A., and Burrill, M. W., *Endocrinology,* 1944, **35**, 187.

[8] Klinefelter, H. F., Jr., Albright, F., and Griswold, G. C., *J. Clin. Endocrinol.,* 1943, **3**, 529.

[9] Jungck, E. C., Maddock, W. O., and Heller, C. G., *J. Clin. Endocrinol.,* 1947, **7**, 1.

[10] Pomerantz, L., and Mulinos, M. G., *Am. J. Physiol.,* 1939, **126**, 601.

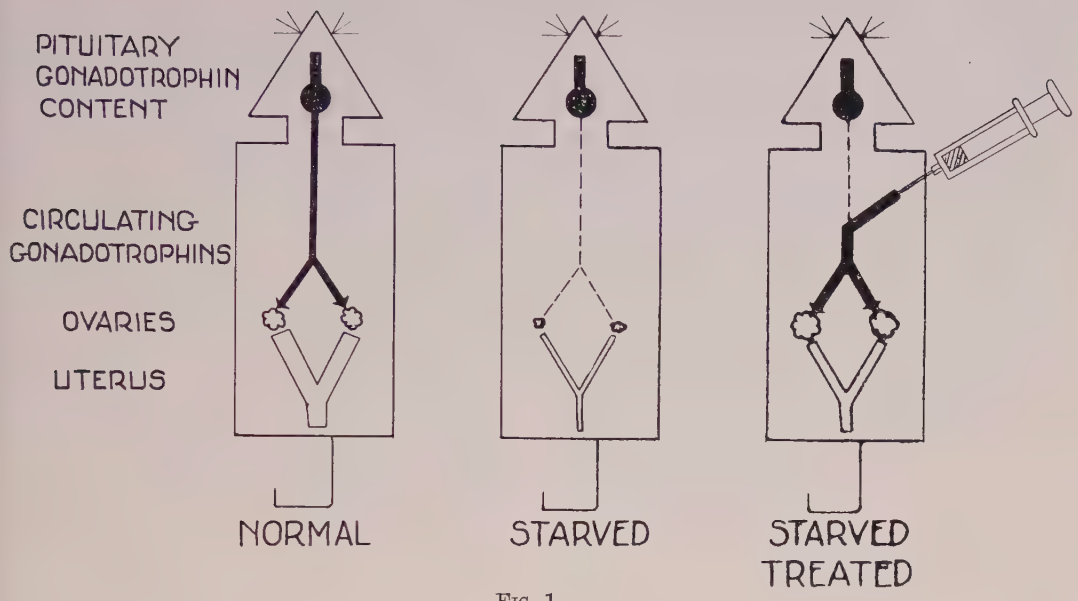

FIG. 1.

anterior pituitary twice a day for 3 days (from the 9th through the 11th day of starvation). A second series of rats (8 months old) were starved for 16 days and compared with fed controls.

Pituitary gonadotrophin content was measured by suspending each anterior pituitary gland in 6.0 cc of water by repeatedly drawing into and expelling from a syringe, then injecting into one 22- to 25-day-old Sprague-Dawley female rat, 1.0 cc twice daily for 3 days. The assay rats were killed 24 hours after the last injection.

Gonadotrophic hormone for injection into the starved adult rats was obtained from the anterior pituitary glands of adult rats castrated 10 months previously. The anterior pituitary glands were suspended in sufficient water so that injecting 0.5 cc twice daily for 3 days equalled administering one half gland.

Results are tabulated in Table I and represented schematically in Fig. 1.

Discussion. The starved rats reacted as if they had been hypophysectomized in that ovaries, uteri and vaginal epithelium underwent marked atrophy. They did not differ from hypophysectomized rats as concerns their potential capacity to respond to exogenously administered gonadotrophins, since administration of rat pituitary suspension, rich in gonadotrophins, restored ovarian, uterine and vaginal cells to normal. Therefore, the conclusion that the circulating blood was low in gonadotrophin content seems warranted.

Pituitary content of gonadotrophin, measured directly, was not below normal. In fact, judged as potency per milligram of gland tissue, the starved rats' pituitary glands (since anterior pituitary weight had fallen circa 40% during starvation) were more potent than normal.

Thus a clear-cut dichotomy between pituitary content of gonadotrophin (normal amounts) and the amount of circulating gonadotrophins (low levels) has been established.

Pituitary content of gonadotrophin is the resultant of the relationship between the amount *produced* and the amount *released*. In acute starvation it is plain that failure of release occurs, as no peripheral gonadotrophin activity is detectable. Production of gonadotrophins must also be decreased since despite the lack of release, excesses are not accumulated within the gland. It is unlikely that the gonadotrophins would remain unchanged within the hypophysis for the entire experimental period of 12-16 days. It is more likely that the release mechanism fails completely and

early in starvation and that the eventual content reflects minimal production.

The mechanism controlling production and release of gonadotrophic hormone during starvation has not been elucidated. The possibility that the amount of circulating gonadotrophins or estrogens are major factors in controlling pituitary content during starvation seems unlikely since gonadotrophin content remains approximately the same despite wide variations in the amount of circulating estrogens and gonadotrophins (high in gonadotrophin injected starved rats, low in starved rats and normal in normal rats).

As a general proposition, release, circulating amount and excretion of gonadotrophins tend to parallel each other. For example, following castration, pituitary content, amount in the circulation and amount in the urine are decidedly greater than for intact animals for all species tested. Following administration of large (unphysiological) amounts of steroid sex hormones to either castrates or normal animals, pituitary content, amount in the circulation and urinary excretion of gonadotrophins fall below normal. Investigation of the 13-lined ground squirrel has revealed that during the period of sexual inactivity (hibernation) gonadal atrophy is associated with decreased amounts of gonadotrophic hormones in the pituitary and in the circulating blood. Just preceding and during the period of sexual activity, there are increased amounts of gonadotrophins in the hypophysis and circulation.[11]

Exceptions to this general proposition, other than the dichotomy found in starvation, have been demonstrated. Lauson, Golden and Sevringhaus[12] observed that immature female albino rats just prior to the onset of puberty had high pituitary levels of gonadotrophins before more than negligible amounts appeared in the circulation. At puberty, release was affected, and with diminishing content, increasing circulating amounts were noted.

Summary. During starvation circulating gonadotrophins fall precipitously whereas the content of hypophyseal gonadotrophin remains as high as under normal circumstances. This is in direct contrast to the usual situation in which pituitary gonadotrophin content accurately reflects the amount released into the circulation.

[11] Moore, C. R., Simmons, G. F., Wells, L. J., Zalesky, M., and Nelson, W. O., *Anat. Rec.*, 1934, **60**, 279.

[12] Lauson, H. D., Golden, J. B., and Sevringhaus, E. L., *Am. J. Physiol.*, 1939, **125**, 396.

16170

In vitro Development of Temporary Penicillin Resistance in Streptococcus Pyogenes.

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The treatment of bacterial infections with penicillin rarely leads to an increase in the resistance of the causative organisms to the antibiotic agent during the course of therapy. While such a phenomenon has been described for *Staph. aureus* and *Strep. viridans*, there is no substantiated evidence that it occurs with *Strep. pyogenes, D. pneumoniae, N. gonorrheae* or other organisms *in vivo*. *In vitro* studies, however, have shown that bacteria may be made less sensitive to penicillin by certain cultural manipulations. Thus, strains of *Staph. aureus*, which were originally susceptible to relatively small doses of penicillin, have been made quite resistant by repeated

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subculture in media containing increasing quantities of this agent. A review of the literature on this subject is not included in this paper; for this, the reader is referred to the recent article of Spink and Ferris.[1]

The development of two kinds of resistance to penicillin in *Staph. aureus* has been demonstrated by Spink and Ferris.[1] The first type is an adaptation that can be reproduced *in vitro* by culturing in media containing increasing quantities of penicillin. The resistance is only temporary, can be abolished by growing the organisms in antibiotic-free broth, and cannot be associated with the production of penicillinase. The second, which occurs in patients as a result of treatment with penicillin, gives rise to strains of *Staph. aureus* in which the resistance is permanent and cannot be decreased by cultural manipulation; these organisms produce penicillinase.

Reports of penicillin resistance in *Strep. pyogenes* are few. One strain of this organism which appeared to be naturally resistant to penicillin (sensitive to 0.625 units per cc) was isolated by Hirsch *et al.*[2] from a patient with scarlet fever. Resistance was present when therapy with the antibiotic agent was first instituted and did not develop as a result of treatment. *Strep. pyogenes* was made resistant to penicillin *in vitro* by McKee and Houck.[3] They decreased the sensitivity of 3 strains of *Staph. aureus*, one strain each of types I, II, and III of *D. pneumoniae* and one strain of *Strep. pyogenes* to penicillin by growing the organisms in broth containing increasing amounts of this drug. The resistance of the beta hemolytic *Streptococcus* was increased 30-fold in a period of 3 months; there was an accompanying loss of virulence. Thirty-two rapid transfers in plain broth or 2 months' storage in the icebox produced no alteration in the degree of acquired resistance. Reduced velocity of growth and variation in colonial form were observed during cultivation in media containing increasing amounts of antibiotic, but the organisms grew luxuriantly after transfer to fresh broth or to blood agar plates which contained no drug. No change in type of enzyme activity was apparent although fermentation reactions were much delayed.

The purpose of the present paper is to report the development of resistance in several strains of *Streptococcus pyogenes* following rapid subculture in broth containing increasing amounts of penicillin and to demonstrate the temporary nature of this resistance as shown by its abolishment by rapid transfer in broth containing no drug. The mechanism of the development of penicillin resistance in bacteria is controversial and will not be discussed here. This subject is reviewed in detail in the papers of Luria,[4] Demerec,[5] and Spink and Ferris.[1]

Methods. Fifteen strains of *Streptococcus pyogenes* recently isolated from the pharynges of patients with scarlet fever were the organisms used in this study. All of the cultures were kept on blood-heart infusion-yeast-tryptose agar and transferred once a week, being kept in the icebox in the interim. Penicillin resistance was determined by the method of Rammelkamp.[6] Solutions of penicillin for incorporation into media were made up freshly each week and kept in the icebox when not in use. The medium in which penicillin resistance was produced consisted of double strength heart infusion-yeast-tryptose broth (0.5 cc) to which was added 0.5 cc of the desired dilution of penicillin and 0.1 cc of a 24-hour culture of *Streptococcus pyogenes*.

All the strains of *Streptococcus* were grown first for 24 hours in broth containing no drug and their resistance to penicillin determined, using an inoculum of between 10,000 and 100,000 organisms per cc. One-tenth cc of a 24-hour broth culture of each was then

1 Spink, W. W., and Ferris, V., *J. Clin. Invest.*, 1947, **26**, 379.

2 Hirsch, H. L., Rotman-Kavka, G., Dowling, H. F., and Sweet, L. K., *J. Am. Med. Assn.*, 1947, **133**, 657.

3 McKee, C. M., and Houck, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33.

4 Luria, S. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 46.

5 Demerec, M., *Proc. Nat. Acad. Sci.*, 1945, **31**, 16.

6 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

inoculated into a tube of broth to which had been added the lowest concentration of penicillin which was inhibitory, as well as into 2 other tubes, one of which contained 20% and the other 40% more drug than the lowest dilution in which the strain grew. The cultures were incubated at 37°C for 24 hours and 0.1 cc of the one showing growth with the largest amount of penicillin was subcultured to 2 other tubes, one of which contained the amount of penicillin which previously had just allowed growth to occur and the other, 120% of this quantity of the drug. This procedure of culturing in the highest concentration of penicillin allowing multiplication of the organisms and in 120% of this amount was repeated every 24 hours except for weekends, when the transfers were made every 48 hours. All cultures showing growth were plated on blood agar every 3 days in order to check the purity of the strains and to determine any changes in colonial or cellular morphology.

After 41 transfers in penicillin-containing broth, 6 strains, the 3 with the highest and the 3 with only moderate increase in resistance to penicillin, were subcultured 16 times more in an attempt to increase to a greater degree their ability to withstand the antibiotic agent.

Following demonstration of penicillin resistance in these 6 strains of *Streptococcus pyogenes* (after 57 transfers), the organisms were cultured in broth containing no drug. Transfers were made every 24 hours and susceptibility to penicillin determined after 10 and 37 subcultures.

Results. The results indicating the development of resistance to penicillin by the strains of *Streptococcus pyogenes* studied are presented in Table I. The data demonstrating loss of resistance to the antibiotic agent following frequent subculture in drug-free broth are shown in Table II.

Determination of penicillin resistance after 41 subcultures in blood-heart infusion broth containing increasing amounts of penicillin revealed 3 strains with no appreciable alteration in their susceptibility to the drug, although they were approximately 2 times more resistant to penicillin than at the beginning of the experiment. This degree of change is within the limit of experimental error and cannot be considered significant. The resistance to penicillin of 4 strains (S-3, S-106, S-46 and S-47) increased 4-fold and of 5 strains (S-1, S-4, S-5, S-7, J-s) 8-fold after 41 transfers in penicillin-containing broth. One strain (S-9) was inhibited only by 16 times and 2 others (S-6, S-48), by 32 times the amount of antibiotic agent which stopped their growth at the start of the study.

Sixteen additional subcultures of 6 of the strains, a total of 57 transfers in drug-containing medium, revealed no further significant increase in penicillin resistance. One strain (S-47) revealed a 2-fold increase but this is within the limit of experimental error and, therefore, probably not significant.

Studies of the colonial and cellular morphology of the organisms at varying times during repeated transfer in penicillin-containing broth revealed no important changes. All of the strains produced matt colonies before and after being made drug-resistant. The colonies of the penicillin-insensitive variants were somewhat smaller than those of the drug-susceptible strains, although they varied considerably in size. Growth in broth and on blood agar plates was less rapid after frequent transfer in penicillin-containing media. No remarkable change in the ability of the *Streptococci* to produce hemolysin after developing resistance to the antibiotic agent was noted. The cellular morphology of the strains, as revealed by the Gram stain, underwent no alteration during the period of decrease in susceptibility to penicillin.

In order to determine whether or not the penicillin resistance which had been produced was temporary or permanent, 6 of the strains which had become relatively insensitive to drug were subjected to rapid subculture (every 24 hours) in broth containing no antibiotic agent. Determination of sensitivity to penicillin was carried out after 10 subcultures and revealed no significant change except with one strain which lost 50% of its resistance; this degree of change is, however, within the limits of experimental error. A

TABLE I.
Development of Resistance to Penicillin by *Streptococcus pyogenes* Following Rapid Transfer in Broth Containing Increasing Amounts of Penicillin.

Strain	Original resistance to penicillin, units/cc	Penicillin resistance after 41 transfers in broth containing penicillin, units/cc	No. of times more resistant to penicillin than original strain	Penicillin resistance after 57 transfers in broth containing penicillin, units/cc	No. of times more resistant to penicillin than original strain
S-1	.0075	.06	8x	—	—
3	.015	.06	4	—	—
4	.0075	.06	8	—	—
5	.015	.125	8	.125	8x
6	.015	.50	32	.50	32
7	.015	.125	8	.125	8
9	.015	.25	16	.25	16
10	.03	.06	2	—	—
106	.015	.06	4	—	—
J-s	.0075	.06	8	—	—
S-46	.015	.06	4	—	—
47	.015	.06	4	.125	8
48	.0075	.25	32	.25	32
49	.015	.03	2	—	—
50	.015	.03	2	—	—

TABLE II.
Loss of Penicillin Resistance of *Streptococcus pyogenes* After Frequent Subculture in Plain Broth.

Strain	Original resistance to penicillin, units/cc	Resistance to penicillin after 57 subcultures in broth containing penicillin, units/cc	Resistance to penicillin after 10 subcultures in plain broth, units/cc	Resistance to penicillin after 37 subcultures in plain broth, units/cc
S-5	.015	.125	.06	.015
6	.015	.50	.50	.015
7	.015	.125	.125	.0075
9	.015	.25	.125	.0075
47	.015	.125	.125	.015
48	.0075	.25	.125	.0075

striking increase in sensitivity to penicillin was present, however, after 37 subcultures in drug-free blood broth; all of the organisms returned to their original degree of susceptibility to the antibiotic. In the transformation from a drug-resistant to a susceptible state there was no change in the colonial or cellular morphology of the bacteria. The penicillin-sensitive streptococci appeared, however, to multiply somewhat more rapidly and to be more hemolytic than the resistant ones.

Summary and Discussion. The resistance of 12 out of 15 strains of *Streptococcus pyogenes* to penicillin was increased by daily subculture (41-57 times) in broth containing increasing amounts of penicillin, the maximum effect being obtained after 41 transfers; no significant increase was noted after 16 additional subcultures. The decrease in susceptibility to the antibiotic agent of 6 of the strains was temporary and was abolished rapidly by culture in drug-free media. No striking effects on the colonial and cellular morphology, degree of hemolysis, or rate of growth could be demonstrated during the process of acquisition or loss of sensitivity to penicillin. The drug-resistant strains, however, grew somewhat more slowly, produced slightly smaller colonies, and were a little less hemolytic than the highly susceptible

ones. No studies of penicillinase production were carried out; in view of the results of Spink and Ferris with *Staphylococcus aureus,* similar investigations on susceptible and *in vitro*-produced resistant strains of *Streptococcus pyogenes* would be very interesting.

Although one strain of *Streptococcus pyogenes,* which was relatively resistant to penicillin, has been described[2] this organism was isolated from a patient before therapy with the antibiotic agent was instituted. There have been no reports of the development of decreased susceptibility to penicillin during treatment of patients with streptococcal (Group A) infections with this drug. Hartman and Weinstein,[7] in a study of over 100 strains of *Streptococcus pyogenes* isolated from cases of scarlet fever were unable to demonstrate any abnormal degree of resistance in the strains originally isolated or in those recovered from patients who had been treated with penicillin and in whom recurrence of *Streptococci* in the pharynx had occurred after therapy was stopped. It would appear from these results, therefore, that the development of resistance to penicillin by *Streptococcus pyogenes* may be easily accomplished *in vitro,* but occurs only rarely, if ever, in patients treated with this agent.

Conclusions. 1. The resistance of *Streptococcus pyogenes* to penicillin has been increased from 4 to 32 times by frequent subculture in media containing increasing amounts of the antibiotic agent.

2. The resistance to penicillin which develops *in vitro* is only temporary and can be abolished fairly rapidly by frequent subculture in drug-free media.

[7] Hartman, T. L., and Weinstein, L., unpublished data.

16171

Temperature Regulation in Albino Rats Correlated with Determinations of Myelin Density in the Hypothalamus.*

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Newborn albino rats are unable to regulate their internal temperatures. When placed in a cold environment their temperatures descend rapidly to that of the environment; a warmer than normal environment results in a marked increase in internal temperature. It has been found that the ability of the young rat to regulate its internal temperature when placed in a cold environment gradually improves from birth to the age of 18-30 days, after which time it usually maintains its body temperature at or very near the normal level.[1]

It has been conclusively demonstrated that the hypothalamus is essential to temperature regulation.[2] Considerable numbers of small myelinated fibers are consistently found in the lateral hypothalamus of the adult brain.[3] In spite of the diverse opinions which have appeared in the literature for many years regarding the correlation of function of neurons with myelination of their axons, it has been considered worthwhile to investigate myelination in the hypothalamus and to attempt correlation of the development of myelin with the beginning of the temperature regulating function of this portion of the brain.

The early literature concerning myelination

* Supported by a grant from the Office of Naval Research.

[1] Hill, R. M., *Am. J. Physiol.,* 1947, **149**, 650.

[2] Ranson, S. W., *Res. Publ. Assn. Nerv. Ment. Dis.,* 1940, **20**, 342.

[3] Ingram, W. R., *Res. Publ. Assn. Nerv. Ment. Dis.,* 1940, **20**, 195.

of nerve fibers and possible correlations between myelination and function has been reviewed by Tilney and Casamajor[4] who also studied somatic activity in the kitten and correlated their observations with the distribution of myelin sheaths in the different fiber paths. They concluded from their investigation that myelinogeny in connection with behavioral components affords strong evidence in the cat that the deposition of myelin is coincidental with the establishment of function in definite fiber systems. They conceded that this coincidence might not, however, be the case in white rats in which both Watson[5] and Donaldson[6] reported complete absence of myelin in the nervous system at birth.

Langworthy[7] studied decerebrate preparations of kittens, young rabbits, and young guinea pigs. He found that myelination in the lower portion of the brain stem and the spinal cord was incomplete in kittens and rabbits in which decerebration was followed by movements of prolonged progression. In more mature animals in which decerebration was followed by the development of typical extensor rigidity, myelination of the long tracts was far more complete. He considered that correlation between myelination of the rubrospinal tract and the occurrence of decerebrate rigidity was strongly indicated.

Langworthy[8] also studied the behavior of pouch-young opossums and attempted to correlate their behavior with myelination of tracts in the central nervous system. He wrote, "The evidence that tracts in the central nervous systems become medullated at the time when they become functional is certainly suggestive, but by no means positive. No one who studies the opossum can evade the fact that this animal is capable of complicated reflex activity before any myelinated fibers are present." Angulo,[9] on the basis of observations of discrete reflexes in rat fetuses, stated that myelination is not a criterion of "functional insulation." Windle, Fish, and O'Donnell,[10] studying cat fetuses of ages from 42 to 60 days after insemination, found a high degree of reflex activity to be present in the fetuses even though the tracts involved were still unmyelinated. They concluded that myelination is not correlated with function in the absolute sense, but conceded that conduction in a given pathway may be improved after myelin sheaths have been acquired.

Ulett, Dow, and Larsell[11] investigated the inception of conductivity in the corpus callosum and the cortico-ponto-cerebellar pathway in young rabbits. They found that myelination of the fiber systems studied is not essential for the conduction of nerve impulses produced by electrical stimulation, and that conduction of such impulses precedes myelination of the fibers by several days in the rabbit. They did, however, observe an increased capacity for conduction in the larger and older animals.

Methods and Materials. Records of the temperatures of rats subjected to an environmental temperature of 5° to 8°C have been obtained through the use of iron constantan and copper constantan thermocouples sealed within the tips of ureteral catheters. Such catheters are easily inserted through the anus and rectum into the colon of rats as young as 4 days of age. The e.m.f. developed by the thermocouple at varying internal temperatures of the animal was originally measured with a Leeds-Northrup potentiometer. Each thermocouple was carefully calibrated with reference to the potentiometer before being used for the recording of temperatures. More recently temperatures have been directly recorded by means of a Brown electronic recording potentiometer. All temperature determinations were made with the

4 Tilney, F., and Casamajor, L., *Arch. Neur. and Psychiat.*, 1924, **12**, 1.

5 Watson, J. B., *Animal Education, Chicago Univ. Contrib. to Phil.*, 1903, No. 4.

6 Donaldson, H. H., *American Textbook of Physiol.*, Phila., 1901.

7 Langworthy, O. R., *Carnegie Inst. of Washington, Contrib. to Embryol.*, 1926, No. 89, **17**, 125.

8 Langworthy, O. R., *J. Comp. Neur.*, 1928, **46**, 201.

9 Angulo, A. W., *J. Comp. Neur.*, 1929, **48**, 459.

10 Windle, W. F., Fish, M. W., and O'Donnell, J. E., *J. Comp. Neur.*, 1934, **59**, 139.

11 Ulett, G., Dow, R. S., and Larsell, O., *J. Comp. Neur.*, 1944, **80**, 1.

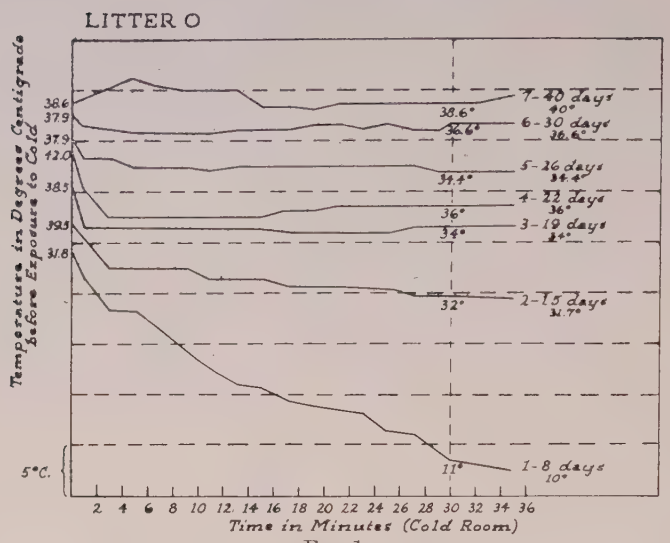

FIG. 1.

Temperature curves obtained from the members of one litter (Litter O) during 35 minutes in the cold room (5°-8°C). The ages (in days) of the respective animals are indicated.

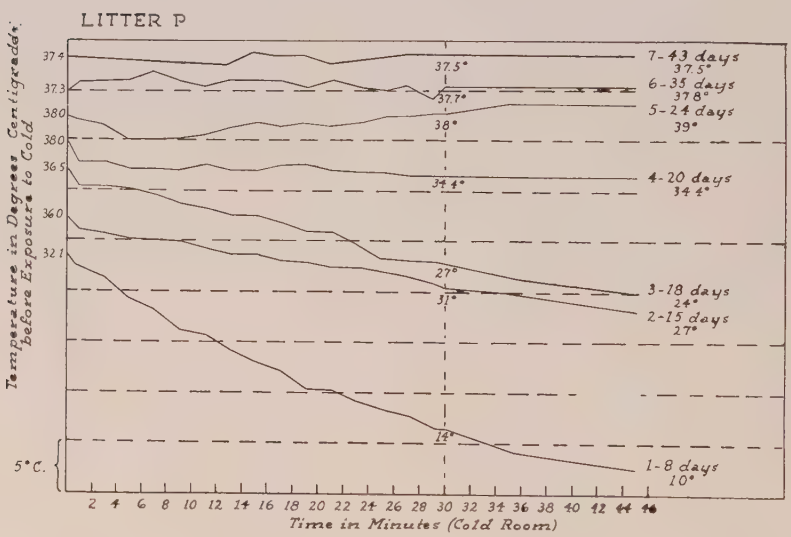

FIG. 2.

Temperature curves obtained from the members of Litter P during 45 minutes of exposure to cold (5°-8°C).

thermocouple inserted to the level of the diaphragm, thus assuring high colonic rather than rectal temperatures. It has been shown that such temperatures more accurately represent the thermal condition of the animal than those obtained by rectum, since the latter fluctuate with changes in the environmental temperature.[1]

The experimental procedure employed in our attempts to correlate temperature regulation with myelination of the hypothalamus has been carried out on twenty litters of Wistar strain rats. When a given litter had reached the age of 6 to 8 days, one animal from the litter was subjected to a cold environment (5-8°C) after first determining

its body temperature in a room whose temperature was approximately 26.5°C. The temperature of the animal was recorded at two-minute intervals with the Leeds-Northrup potentiometer, or at one-minute intervals with the Brown electronic potentiometer. Temperatures were recorded during 30 to 45 minutes. In the preparation of Fig. 3, 4, and 5, the temperatures after 30 minutes in the cold room were used to illustrate graphically the difference between normal environmental temperatures and those resulting from exposure to cold. Following exposure to cold, the animal was anesthetized with ether and perfused with one of three fixatives—10% neutral formalin, Erlicki's solution (copper sulphate and potassium bichromate), or 3½% potassium bichromate. The first of these preceded the Weil stain for myelin,[12] the second is the fixative recommended by Kultschitzky for his myelin stain,[13] and the third is that found most satisfactory for the myelin stain used by Ulett and his collaborators.[11] After perfusion, the brain was removed, fixation was continued for the specified period, and in the solutions specified by the originator of the stain. A block from the center of the brain which included the hypothalamus, or a complete half brain was finally imbedded in paraffin, sectioned, and mounted on slides. Other animals from the same litter were similarly treated at appropriate intervals until the litter was exhausted. The same fixatives were applied in a uniform manner to the brains of all the members of a given litter. When all the hypothalami from a given litter had been sectioned at as uniform a thickness as is possible with the Spencer rotary microtome and placed on slides, staining of the sections was carried out. In order that comparisons based on the density of the myelin stain could be made between the hypothalami of the rats of different ages, three slides (9-12 sections) were selected from each animal in the litter. These were placed in a staining rack and stained en masse. Theoretically, if

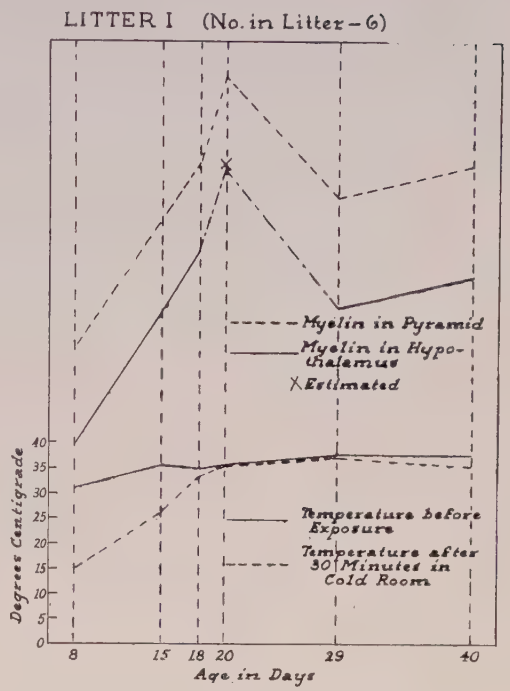

FIG. 3.

Records of temperatures before and after 30-minute exposures to cold and curves representing the density of myelin staining in the pyramid and lateral hypothalamus as determined photometrically. (Technical difficulties, resulting in loss of sections of the hypothalamus in the 20-day animal, made an accurate determination impossible.)

the stain is specific for myelin, those hypothalami containing the least myelin (or no myelin) should be less deeply stained than those in which myelination is further advanced. In our experience the myelin stain reported by Ulett, Dow and Larsell[11] has given the most satisfactory results, since the nerve cells remain almost entirely unstained in sections prepared according to their method.

Determinations of staining density have utilized a photometric method. The light from a Spencer microscope lamp has been directed through a monochromator to the substage of a binocular microscope; the monochromator was set to deliver light at a wavelength of 600 mu. The phototube of a Photovolt electronic photometer is firmly attached to the right ocular of the microscope in such a manner that all light traversing that ocular

12 Weil, A., *Arch. Neur. and Psychiat.*, 1928, **20**, 392.

13 Lee, B., *The Microtomist's Vade-Mecum*, 10th ed., p. 525.

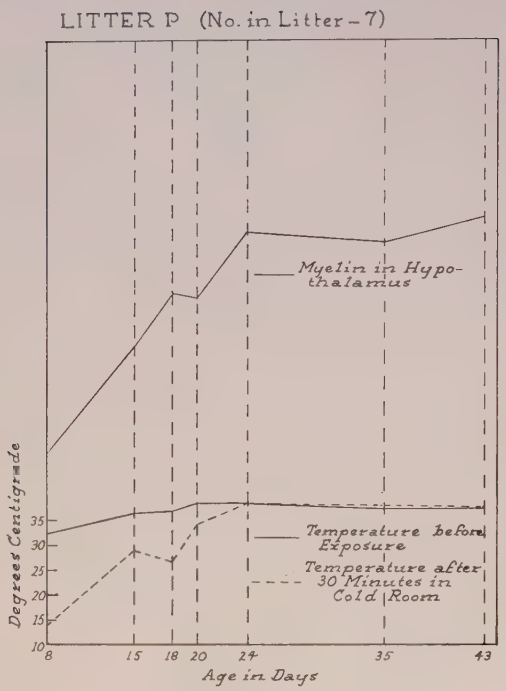

FIG. 4.

Records of temperatures before and after 30-minute exposures to cold and curve representing the density of myelin staining in the lateral hypothalamus in Litter P. (*Cf.* Fig. 2.)

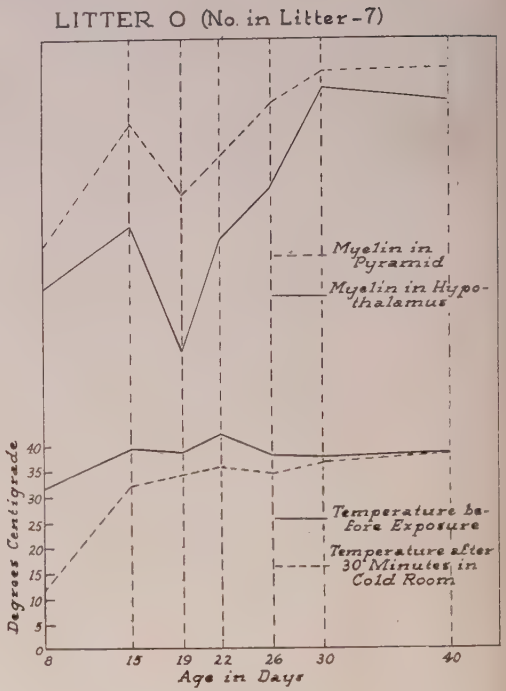

FIG. 5.

Records of temperatures before and after 30-minute exposures to cold and curves representing the density of myelin staining in the pyramid and lateral hypothalamus in Litter O. (*Cf.* Fig. 1.)

is focused upon the photoelectric cell and thus recorded directly upon the galvanometer of the instrument. The procedure for obtaining the density of the myelin stain in a given section consisted of focusing upon the lateral hypothalamic area through the left ocular of the microscope after which the slide was moved to an area free of sections; without altering the focal adjustment of the microscope, the left ocular was covered and the light entering the microscope was adjusted by means of a rheostat and the iris diaphragm at the inlet of the monochromator to give an arbitrary galvanometer reading, which "zero" reading was then kept constant for an entire series of determinations. The maintenance of a constant "zero" point on the scale compensated for differences in thickness of slides, cover slips, and mounting media. After the light had been adjusted, the section was moved back into the field, the left ocular was again covered, and the galvanometer reading recorded. The difference between this reading and the "zero" reading represented the amount of light absorbed by the stained section. This procedure was repeated on several sections of the hypothalamus of a given animal, and the several readings were then averaged. The averages were used in the construction of myelin curves for the litter being studied. Typical myelin curves obtained by this method in three of the litters investigated are shown in Fig. 3, 4, and 5. Myelin determinations have also been made on the pyramid of the medulla in several of the litters studied (Fig. 3 and 5) in order to check myelination in an area completely free of nerve cell bodies.

Results. The temperature curves during exposure to cold in animals from the same litter at progressively older ages are illustrated in Fig. 1 and 2. These are entirely consistent with previous obervations by one of us[1] and show a progressive improvement in ability to regulate against cold. Regulation, aside from an initial drop of some 3½ degrees, was fairly well established in animal 3 (Fig. 1) at 19 days of age, and was completely established

in animals 6 and 7 at 30 and 40 days of age. Regulation in Litter P (Fig. 2) was quite good in animal 4 at 20 days of age, and was completely established in animal 5 at 24 days of age.

In the majority of litters studied, very adequate regulation against cold was evident at 18 and 20 days of age (Fig. 3). In some litters complete regulation appeared later (24 days in Litter P, Fig. 4, and at 30 days, Litter O, Fig. 5). The density of myelin stains as measured by the method described above has regularly reached a high level coincident with the beginning of regulation (Fig. 3, 4, and 5). In most of the litters studied, there has been a tendency for a levelling off or a depression of the myelin curve during the post-regulatory phase. As was to be expected, the pyramids of the medulla showed considerably greater degrees of staining density than the hypothalamus at all ages. There was, however, a striking parallelism between the curves representing density of the pyramids and of the hypothalamus in progressively older rats (Fig. 3 and 5).

The marked pre-regulatory depressions in the myelin curves for hypothalamus and pyramid in Litter O (Fig. 5) may have been due to a dietary deficiency, since we were unable to obtain lettuce for our rat colony at the time when this particular litter was born. The same dietary deficiency may have been responsible for the delay in attainment of regulatory ability and the corresponding delay in myelin formation as indicated by the fact that the curves reached their highest level in the 30-day animal.

Discussion. Koch and Koch[14] studied the chemical differentiation of the brain of the albino rat during growth. Their analyses were carried out on whole brains of rats 1, 10, 20, 40, and 120 days of age. The results of their investigation so far as phosphatides are concerned may be summarized as follows:

Age of rats in days	1	10	20	40	120
Phosphatides	15.2%	12.3%	21.4%	21.8%	21.6%

The phosphatides have been selected from their table as being the most likely substances having affinity for the various modifications of the Weigert stain for myelin which have been used in our experiments. The rather marked increase in phosphatides during the first 20 days compares favorably with the curves which have been obtained by our method of determining the concentration of myelin in the hypothalamus and pyramid of the albino rat. None of our investigations of myelin have been carried out on rats younger than 6 days of age, and we therefore have no material available for comparison with the figure of 15.2% phosphatides found in rats one day of age. It will be noted that Koch and Koch actually found a lower percentage of phosphatides in the brains of 10-day rats than in those one day old. The tendency for our myelin curves to flatten out or to be depressed during the period between the 20th and 40th days of life may possibly be due to the failure of phosphatides to increase appreciably during that period. Further studies are in progress to determine, if possible, the individual specificities of our myelin stains for lecithin and cephalin. Cholesterol will also be studied in this connection. Langworthy[15] called attention to the well known fact that animals, particularly those born in large litters, vary greatly in their maturity at birth and in the rapidity of their growth thereafter. We have studied litters of albino rats containing as many as 12 individuals, and have found that the attainment of the ability to regulate internal temperature when exposed to a cold environment is somewhat delayed in such large litters and that there is a tendency for the myelin curves to be much less steep in the period between 6 and 20 days than is the case in the smaller litters.

Watson[5] stated that myelination does not begin in the higher centers of the brain until rats have attained the age of 24 days. Although this may be true of cortical association fibers, it certainly does not appear to be true of the hypothalamus, if our method is valid for the determination of the presence of myelin.

[14] Koch, W., and Koch, M. L., *J. Biol. Chem.*, 1913, **15**, 423.

[15] Langworthy, O. R., *Carnegie Inst. of Washington, Contrib. to Embryol.*, 1929, No. **114**, **20**, 127.

The somewhat earlier development of resistance to cold in these animals (Wistar strain) as compared to those previously studied (Denver University strain) may be due to the difference in strain, or it may be due to the fact that in the earlier experiments lettuce was not a part of the diet of the mothers whereas it was in these experiments in all cases except Litter O (Fig. 5).

Conclusions. The optical densities of sections of the hypothalamus stained by modifications of the Weigert method have been measured in rats varying in age from 6 to 70 days by means of monochromatic light and an electronic photometer. These measurements have been correlated with the abilities of the animals to regulate their internal temperatures when exposed to a cold environment. We believe that our method has recorded densities which are dependent mainly upon the amount and/or character of the myelin in the hypothalamus at the respective ages studied, and that the increase in myelin from birth to 20 days of age may contribute to the acquisition of the capacity for temperature regulation. We do not believe that myelination in the hypothalamus is the only factor concerned in the acquisition of this function. Many other factors are no doubt operative and many of these are currently being investigated.

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Teratogenetic and Lethal Effects of Influenza-A and Mumps Viruses on Early Chick Embryos.

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Gregg[1] demonstrated a high incidence of congenital malformations in children whose mothers had contracted rubella (German measles) during the early months of pregnancy. These observations were confirmed by Swan[2,3,4] in Australia, and by American and European workers. Over 400 cases are now on record. The most common abnormalities caused by rubella are: cataract in one or both eyes, heart defects, microcephaly and deaf-mutism. The incidence of malformations following infection of the mother within the first 2 months of pregnancy is almost 100%, but low after the 4th month. It seems that a very early infection (around 1-1½ months of pregnancy) causes predominantly cataract, whereas a later infection (around the 2nd month) causes predominantly deaf-mutism.

The discovery that the rubella virus passes through the placenta and causes a specific pattern of localized malformations in the human embryo is of great interest from the point of view of embryology, virus biology and clinical obstetrics. It seemed to be of importance to analyze the effects of viruses as teratogenetic agents under the controllable conditions of animal experiments.

The chick embryo suggested itself as a suitable embryonic material, since it is known

* Special Research Fellow of the National Institute of Health.

† Surgeon, National Institute of Health.

‡ Supported in part by a grant from the Rockefeller Foundation. The authors wish to acknowledge the valuable assistance of Mr. Li-Hua Kung.

1 Gregg, N. McA., *Trans. Ophthalmological Soc. of Australia,* 1941, **3**, 35.

2 Swan, C., Tostevin, A. L., Moore, B., Mayo, H., and Barham Black, G. H., *Med. J. Australia,* 1943, **2**, 201.

3 Swan, C., Tostevin, A. L., Mayo, H., and Barham Black, G. H., *Med. J. Australia,* 1944, **1**, 409.

4 Swan, C., and Tostevin, A. L., *Med. J. Australia,* 1946, **1**, 645.

that the tissues and the extra-embryonic membranes of the chick embryo are a favorable medium for the culturing of a number of viruses (Beveridge and Burnet[5]). Furthermore, the absence of a placenta in the chick makes a direct infection of the embryo possible, thus avoiding the complications introduced by an extra-embryonic barrier.

The rubella virus was not available. Instead, we used influenza-A virus (strain PR8) and mumps virus. Experiments with rubeola virus are in progress.

Developmental stages used. The routine methods of culturing viruses in eggs make use of chick embryos incubated for 8 or more days. In these stages the chief reservoirs for virus, namely, the allantoic and amniotic sacs are well developed. However, embryos of that age would be unsuitable for our purpose since most of their organs are far advanced in their differentiation. Teratogenetic effects can be expected only if the embryos are infected at much younger stages, that is, in the first phases of morphogenesis or in the still earlier stages of organ determination.

With the exception of a group of experiments on 4-day embryos all embryos used were incubated for approximately 48 hours in a forced-draft incubator at a temperature of 98°F. The youngest embryos had about 16 somites; their eyes were in the optic vesicle stage; the axis was straight and the heartbeat had just begun (Arey;[6] Fig. 508B). The oldest embryos had about 27 somites; their eyes were in the optic cup stage with lenses; 3 pairs of visceral arches were developed; the cranial and cervical flexures of the head and the torsion of the main axis were in progress; the amnion had overgrown the head approximately to the heart level (Arey;[6] Fig. 523B). Most embryos were in stages intermediate between these 2 (Arey;[6] Figs. 506C and 523B). All eggs came from a Government controlled flock of New Hampshire Reds.

Technique of infecting 48-hour embryos. The egg was candled, and the position of the blastoderm was marked on the shell (the embryo is not yet recognizable at that stage). The egg was then placed on a Syracuse dish with a cotton-cushion. A square window, about ¼ inch in length and width was sawed in the shell over the blastoderm, using a hacksaw blade. The window was removed and the underlying shell membrane was ruptured after it had been thoroughly moistened. The embryo was thus exposed. Next, the transparent vitelline membrane which surrounds the entire yolk was ruptured over the embryo with a fine glass needle or a pair of watchmaker forceps. (Instruments described in Hamburger[8]). This membrane had been found to be a barrier to virus infections. Meanwhile, a tuberculin syringe (½ cc) with a 27-gauge needle had been filled with the standard dosage of 3/100 cc of the solution to be injected. The point of the needle was inserted through the hole in the vitelline membrane, and the liquid spread slowly over the embryo. Care was taken to hold the needle point close to the embryo but not to touch it nor to inject abruptly in order to avoid injuries to the embryo or blastoderm. The window was then closed by sealing with paraffin the piece of shell which had been previously removed. Incubation was continued in a Buffalo incubator without forced circulation, running at 99°-100°F. All instruments, glassware, and fluids were kept sterile. All eggs were candled each day.

Experiments with Influenza-A virus. Source of virus material. An egg-adapted PR8 strain of influenza-A virus was used. Each sample used had been tested for virus titer by Salk's modification of Hirst's red cell agglutination test, cultured for sterility and stored in flame-sealed ampoules in a frozen state at –73°C. The 4th to 7th egg-passages (p4 to p7) were used in our experiments. They had titered as

5 Beveridge, W. I. B., and Burnet, F. M., *Medical Research Council,* 1946, Special Report Series No. 256.

6 Arey, L. B., *Developmental Anatomy,* Fifth edition, 1946, W. B. Saunders, Philadelphia, Pa., and London.

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TABLE I.
Infection of 2-day Chick Embryos with Influenza-A Virus.

Exp.	Passage	Concn.	Total infected	Discarded	Revised total	Abnormal	Normal	Died, days after infection
5fl	p^4 *	undil.	20	—	20	20	—	1 -2½
6fl	p^4	1:4	23	5	18	18	—	1½-2
8fl	p^5 †	1:10	25	1	24	11	13	1½-2
10fl	p^5	1:100	25	1	24	3	21	12 were alive at 18 days
14fl	p^5	1:4	28	3	25	25	—	1½-2½
15fl	p^6	1:10	22	4	18	18	—	1 -2
16fl	p^6	undil.	9	—	9	8	1	½
26fl	p^6	1:4	10	1	9	7	2	2 -2½
27fl	p^6	1:20	10	2	8	7	1	1½-2½
28fl	p^6	1:50	9	—	9	9	—	2½-3
30fl	p^7	1:4	48	1	47	47	—	1 -2
34fl	p^7	1:100	12	—	12	12	—	½-1
35fl	p^7	10^{-3}	12	—	12	11	1	1 -2
36fl	p^7	10^{-4}	12	1	11	11	—	2 -3
37fl	p^7	10^{-5}	12	—	12	12	—	2 -3
38fl	p^7	10^{-6}	12	—	12	12	—	2 -3
39fl	p^7	10^{-7}	12	1	11	2	9	6 were alive at 5½ days
47fl	p^7	1:100	10	—	10	9	1	2 -3
48fl	p^7	1:100	8	—	8	8	—	1½-3
51fl	p^7	1:100	20	—	20	20	—	2 -3
52fl	p^7	1:10	20	1	19	19	—	1 -2½
Total			359	21	338	289	49	½-3

* Bracket indicates that the same sample was used.
† Sample had probably low virulence: see text.

follows (before freezing): p4 at 1:16000; p5 at 1:2048; p6 at 1:32000; p7 at 1:8000.

Experimental results. Virus-infected allantoic fluid was used in concentrations from undiluted to 10^{-7}, as indicated in Table I. With the exception of 2 series, practically all embryos infected with dilutions up to 10^{-6} died within 3 days after infection (5 days of incubation), and showed severe abnormalities. The 2 exceptional series (8fl and 10fl) had been infected with the same sample of p5 diluted to 1:10 and 1:100 respectively. As was mentioned above, p5 had the lowest hemagglutination titer of the passages used. Since all other passages gave almost 100% lethal and teratogenetic effects at the same dilutions, it is assumed that this particular sample had an exceptionally low virulence. The predominance of normal embryos in 39fl indicated that the threshold of infectiousness is reached at a dilution of 10^{-7}. If all experiments with the exclusion of 8fl, 10fl, and 39fl are taken together, the incidence of lethal and teratogenetic effects amounts to almost 98% (273 of 279 cases). The 6 normal specimens have probably escaped an infection due to a faulty technique.

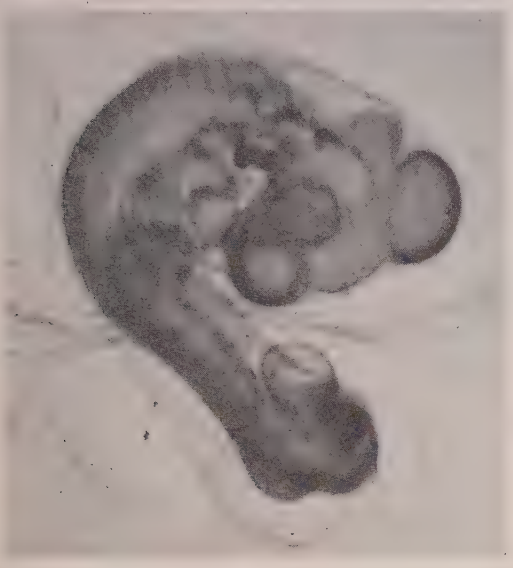

FIG. 1.
Normal 4-day chick embryo.

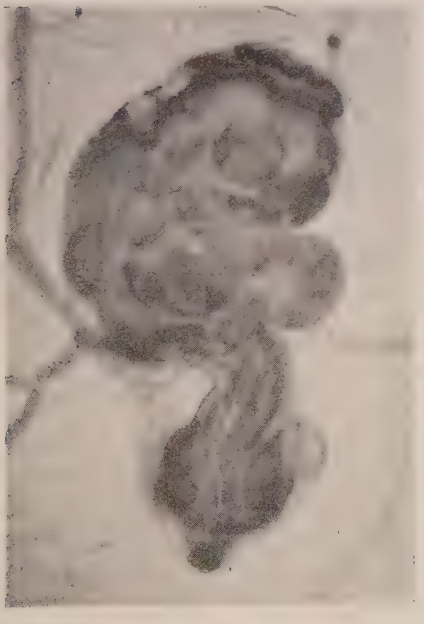

FIG. 2.
Case 15fl-8. Infected with influenza-A virus. (PR8, p6, allantoic fluid diluted 1:10) at 2 days of incubation. Fixed at 4 days of incubation. Stained with Delafield's Hematoxylin.

FIG. 3.
Case 15fl-4. Same as Fig. 2. Fixed at 3½ days of incubation. Stained with Delafield's Hematoxylin.

Description of abnormalities. All infected embryos were severely malformed. The following symptoms were observed macroscopically (see Figs. 2, 3). A histological exam-

ination of the material is in progress.

(1) The head and, in particular, the brain, are disproportionally small (*microcephaly, micrencephaly*). Tel-, di-, and mesencephalon are abnormal in shape. This is particularly striking in the case of the mesencephalon which is a large, protruding thin-walled vesicle in normal 3- to 5-day embryos, whereas in all infected embryos it is a flattened and unexpanded structure, showing a ring of whitish tissue at its base. The eyes seem to be less affected. They are pigmented in the older embryos and approach normal size which makes them appear disproportionally large. No striking gross abnormalities were found at the trunk and tail levels and in the limb buds.

(2) The main axis is more or less twisted in all infected embryos. In normal development, conspicuous flexures occur during the stages under consideration. First, a torsion of the head takes place in such a way that the left side faces the yolk. Simultaneously, the head undergoes a bending in the midbrain level (cephalic flexure) which brings the forebrain in a position almost parallel to the hindbrain. Shortly afterwards, a bending in the hindbrain level (cervical flexure) takes place. An additional temporary flexure occurs in the wing level which continues into the trunk level. As a result, an embryo of about 27 somites has the appearance of a question mark (?). By the 4th day, the flexures have been smoothed out and the dorsal axis forms a smooth circular curve (Fig. 1). In the infected embryos, the cervical flexure and the one in the wing level are more or less strongly accentuated. In the most severe cases, the main axis is folded upon itself, forming a tight sigmoid curve. There may be one or 2 twists in the same embryo. The points of excessive twisting vary from case to case, but they are always in the cervical, wing, or middle trunk levels.

(3) The amnion which grows out as a protective membrane during the stages following infection is severely impaired in its growth. The head fold of the amnion usually proceeds to cover the head, but the lateral and tail folds either fail to grow out altogether or they do so at a greatly reduced rate. As a result the amnion rarely closes over the embryo, and gaps were found ranging from a small hole over the posterior end to the complete exposure of the embryo from the heart level to the tail end. In the few cases in which the amnion does close, it is very tight, the amniotic fluid is under high pressure, and, as a result, the lateral body walls which are continuous with the amnion are folded dorsad instead of ventrad, exposing the viscera, such as mesonephros and intestine (*ectopia viscerum*). It is possible that the severe twists of the main axis are wholly or in part, secondary effects of this failure of amniotic growth. The rapidly growing and stretching embryo seems to be crammed for space in the amniotic cavity; the underdeveloped amnion would offer a barrier, and the result would be a passive folding of the axial organs at the points where normally a slight bending occurs.

(4) The growth of the entire embryo seems to be impaired; however, this point requires verification by exact measurements.

Each symptom showed a moderate range of variability. However all symptoms appeared in all 273 affected specimens. The uniformity and reproducibility of the syndrome under the standard conditions of the experiment were striking. The circulatory system did not seem to be affected. The allantoic vesicle grew out normally in most instances.

The lethal effect seems to be an all-or-none effect in the sense that embryos which receive an infectious dose (dilutions to 10^{-6}) die within 3 days after infection, whereas embryos showing no abnormalities and surviving the 3rd day seem to have remained uninfected. Within this 3-day range, there seems to be a correlation between virus concentration and life span. In a dilution test, 6 sets of embryos (12 per set) were infected with serial 10-fold dilutions from 10^{-2} to 10^{-7} of the same sample. The embryos infected with a dilution of 10^{-2} died within one day after infection, those infected with 10^{-3} died within 1-2 days and those infected with 10^{-4} to 10^{-6} died within 2-3 days. The cause of

TABLE II. Control Experiments.

Type	Exp.	Conc.	Total	Discarded	Revised total	Died, days after 2-day stage		Alive, when opened, days after 2-day stage			Abnormal†	
						1-5	5½-12	1-5	5½-12	more than 12	Total	%
Untreated	49c	—	72	5*	67	3	1			63	3	4.5
0.9 saline	32c	—	54	1	53	9	13	2	1	28	2	5.8
Allantoic fluid	5cfl	undil.	19	1	18	2	—	16	—	—	—	
	8cfl	1:10	14	—	14	3	2	1	1	7	—	
	12cfl	undil.	16	1	15	5	—	4	—	6	1	
	15cfl	,,	15	4	11	7	—	2	—	2	4	
	17c	,,	24	3	21	9	5	—	1	6	1	
	18c	,,	24	—	24	9	3	3	—	9	2	
	19c	1:4	7	—	7	3	—	—	1	3	—	
	20c	undil.	15	1	14	4	2	—	8		1	
	21c	,,	15	1	14	4	4	—	6	—	2	
	22c	,,	24	3	21	14	2	—	5	—		
Total: allantoic fluid:			173	14	159	60	18	26	22	33	11	6.9
Ultraviolet irrad. virus	25c	undil.	32	4	28	11	5	2	—	9	2	7.1

* Sterile.

† All dead at 3-6 days of incubation.

death is obscure. As was mentioned, the circulation seems to be intact; there is evidence of a generalized infection.

Control Experiments (Table II). In order to test the incidence and types of abnormalities occurring in the egg material used for our experiments, 72 unopened eggs were incubated. Of these 5 were sterile, 3 were found dead and abnormal at 3½ days, one died at 12½ days and the remaining 63 embryos were opened and found to be normal on the 15th day of incubation. Of the abnormal embryos, one showed a twist in the cervical region and a slight microcephaly. However, the presence of a fully expanded midbrain, and other details, distinguish this embryo distinctly from the influenza-infected embryos. Another specimen had head abnormalities similar to the first one but no twist. In the third, the limb buds were bent upward, and the posterior trunk axis bent ventrad.

Injections of 0.9% salt solution resulted in an increase of the mortality during the early stages of development. The percentage of abnormalities was not higher than in untreated embryos. The 2 abnormal embryos of this series had died between 2 and 3½ days after treatment. They both had twists of the axis, and head abnormalities similar to those found in the untreated controls but different from those described for influenza-infected embryos.

Injections of uninfected allantoic fluid from normal 15-day embryos resulted in a slight increase in the incidence of abnormalities and in a marked increase of the mortality in early stages. Of the 11 abnormal embryos, 8 showed slight to severe twists of the axis. Malformations of the head were found twice in combination with twists, and twice in otherwise normal embryos. They were, again, of a type similar to those occurring in other controls but different from those found in virus-infected embryos. Furthermore, not a single case in any of the control series presented the combination of head, axis, and amnion deficiencies which is characteristic of all influenza-infected embryos. Hence, the symptoms described above must be considered as specific effects of the virus infections. The

fact that some of the abnormal control embryos exhibit a twist of the axis similar to infected embryos is readily understood; the virus infection interferes with the normal developmental processes at certain points of least resistance which are equally vulnerable to other teratogenic agents.

Evidence for virus multiplication in infected embryos. The following experiment shows that living virus and not its toxic products are instrumental in bringing about the teratogenetic effects. Samples of allantoic fluid (p5 and p6) virus were inactivated by ultraviolet light. The irradiated fluid was used undiluted for the injection of 28 embryos. Two abnormal embryos were found at 4 days of incubation (7%); both showed twists, and the one, in addition, a severe deformity of the hind end. The percentage of abnormalities and the mortality rate at early stages up to 12 days, were almost identical with those of the normal allantoic-fluid controls. The experiment shows that the characteristic effects of influenza-A virus are checked, if the virus is killed. Evidence that virus growth had taken place following infection was shown with 10 specimens of Exp. 30fl (Table I) collected when alive 2 days after infection and showing severe symptoms. They were removed from the blastoderm, pooled, ground in the Waring Blender and the supernatant titrated from 10^{-1} to 10^{-7} in 8-day embryos. The allantoic fluids harvested from the survivors after 6 days' incubation tested for hemagglutination indicated a titer of at least 10^{-7}. The actual titers were probably higher since one cc of saline per embryo was added before the dilutions were made. In addition a hemagglutination titer was made with the original embryonic tissue and was found to be 1:1024. Previous experiments have shown that the egg-passage strain of Influenza-A virus dies off rapidly in the liquid state when kept at room temperature. It is concluded that the virus present in the infected 4-day embryos is not merely due to survival of injected virus particles but that it is the result of multiplication.

Infection by transplantation of infected tissues. As was shown, the head, and, in particular, the brain are severely malformed, whereas the trunk and tail rarely show macroscopic defects. Since care was taken to obtain an equal spread of the infectious fluid, this differential effect seems to be due to inherent differences in tissue susceptibility. An attempt was made to alter the pattern of abnormalities, by means of the method of embryonic transplantation which allows one to bring infected tissues in close contact with any part of the embryo. The following technique was employed: Highly abnormal embryos were obtained by injecting allantoic fluid (p7), diluted to 1:4 and 1:100 respectively. A number of embryos were recovered when alive, at 4 days of incubation. They were removed from the blastoderm, transferred to a Syracuse dish, and small pieces of the midbrain or other brain parts were cut out with an iridectomy knife. These fragments were then implanted in different levels of normal 2-day embryos, that is, near the brain, near trunk somites, or in the hind limb or tail region (for further details of technique see Hamburger[8]). The host embryos were incubated for another 2 days. All embryos (altogether 26 cases) showed the characteristic symptoms of influenza-A virus infection, including microcephaly, no matter where the transplants had been placed. Some transplants had fused with the host tissues, for instance with the left hind limb bud, or with somites; others were found floating freely near the embryo, still others were not recovered at all. Control experiments with normal brain tissue gave no abnormalities. These experiments give further evidence of the presence of living virus in the brain tissue of infected embryos. Since the host embryos failed to show focal infections at the posterior parts of the body, following implantation of infected tissue in these levels, we assume that the virus has spread from the transplants to all parts of the body and that a selective infection of the brain has taken place. It seems that the embryonic brain offers a preferential medium for influenza-virus growth. Further direct evidence for a higher rate of virus multiplication in the anterior parts of the embryo as compared

to the posterior parts was obtained by titrating the emulsified anterior and posterior halves of 18 infected embryos by the hemagglutination technique. The anterior halves titered to 1:6400 and the posterior halves to 1:1600.

Infection of 4-day embryos with Influenza-A virus. In a preliminary experiment (23fl) a dose of 3/100 cc per embryo of allantoic fluid (p6, diluted 1:4) was injected directly into the extra-embryonic coelom, through a hole in the somatopleure between the wing and leg buds, after a window was sawed over the embryo, the latter placed on the candle and the syringe needle inserted through the slit for about one-half its length and aimed at the embryo. Since both techniques gave identical results, the latter, more simple procedure was used henceforth. Of 84 injected embryos, 74 were found dead between one and 3½ days after infection; the others were opened when alive for the purpose of preservation. The majority of the embryos appeared to be without gross abnormalities but a few exhibited slight twists of the axis, and a few others showed signs of brain infections.

Dilution tests were run parallel with those reported above for 2-day embryos, and again a correlation was found between concentration and life span. Even a dilution of 10^{-7} which was not fatal for 2-day embryos killed 7 of 8 four-day embryos within 3½ days after infection. It is clear from these experiments that 4-day embryos are at least as susceptible as 2-day and 8-day embryos. The absence of abnormalities in infected 4-day embryos is understandable since at the stage of injection the development of organs is advanced far beyond the 2-day stage, and the amnion is closed so that no twist of the main axis of the embryo should be expected if our explanation of the mechanics of twisting given above is correct.

Experiments with Mumps Virus. Source of the virus material. The material for one experiment (15mp) was derived from strain "M" (Habel[7]) which had been carried through 37 monkey-parotid passages and then established in the allantoic sac of the chick embryo. Injections for routine passages were made in 8-day embryos (2/10 cc per embryo) and

TABLE III.
Infection of 2-day Chick Embryos with Mumps Virus.

Exp.	Passage and strain	Conc.	Total	Died, days after infection							Preserved: alive 1-5 days after injection	Abnormalities		
				1	2	3	4	5	6	Total		Twists	Other	Total
12mp	4(EVI)	undil.	18	—	1	—	—	13	—	14	4	2	—	2
13	4 ,,	,,	19	1	—	2	—	9	—	12	7	—	—	
14	4 ,,	1:10	13	—	1	4	—	3	—	8	5	1	1	2
4	6 ,,	,,	13	1	—	3	5	2	—	11	2	—	1	1
11	7 ,,	undil.	7	—	2	2	2	—	1	7	—	—	2	2
17	13 ,,	,,	27	1	1	2	1	1	—	6	21	5	2	7
18	13 ,,	1:10	18	1	3	6	1	—	—	5	7	—	—	
19	13 ,,	1:100	7	1	2	—	2	—	—	5	2	—	—	
15	38(M)	undil.	12	2	6	2	—	1	—	11	1	7	0	7
			—	—	—	—	—	—	—	—	—	—	—	—
		Total	134	7	16	21	11	29	1	85	49	13 = 11.2%	6	21 = 15.7%
Ultraviolet irrad. virus											5			
16emp	13(EVI)	undil.	28	—	1	2	2	2	—	7	16 alive 11 days after injection			

the allantoic fluid harvested on the 18th day. The sample used in 15mp represents the 38th allantoic passage of "M." In all other experiments a more virulent strain was used which was originally derived from "M." It multiplied more rapidly in eggs and titered higher on hemagglutination and will be designated as "EVI." The samples of "EVI" used in our experiments came from allantoic sac passages 4, 6, 7, 13.

The technique of infection was the same as that used for influenza-A, the embryonic stages and the standard dose being the same.

Results. Of 161 infected embryos, 134 survived the operation (tabulated in Table III). Of these, 49 were preserved when still alive. The table shows that the mumps virus is lethal within 5 days after the infection of 2-day embryos. This effect is convincingly demonstrated by those experimental series in which all, or all but one or 2 specimens, had been allowed to continue development until death occurred. The 49 specimens which had been fixed during the first 5 days when still alive were unselected material. The percentage of abnormalities (15.7%) were higher than in allantoic fluid controls (6.9%). Twists of the axis were the most common malformations; they occurred with a particularly high incidence in Exp. 15mp in which the "M" strain was used. No case of microcephaly was observed. The wing and leg buds were abnormal in 4 cases, and a shortening of the beak was found in a few instances.

Evidence for virus multiplication in infected embryos. Infected allantoic fluid (16cmp) inactivated by ultra-violet light produced no higher mortality than normal allantoic fluid. Sixteen of 28 injected embryos were alive and normal when examined on the 11th day of incubation. We conclude that the lethal effect must be attributed to the presence of living virus.

Six embryos (of series 18mp) which were alive when recovered on the 4th day after infection were used for titration in 8-day embryos, the dilutions ranging from 10^{-2} to 10^{-7}. After 7 days' incubation hemagglutination showed the titer to have been 10^{-6}. Furthermore, the allantoic fluid and the emulsified tissues of the 6 original embryos of 18mp were tested by hemagglutination. The allantoic fluid titered to 1:32, the tissues were negative. The latter results correspond to those of Habel[7] where the allantoic fluids titered much higher than the tissue emulsions. Since it is known that mumps virus contained in allantoic fluid in a liquid state is completely inactivated within 48 hours at room temperature, we conclude that virus multiplication has taken place in our experiments.

The results obtained with mumps virus are of interest in two respects. First, Habel[7] had found that the infection of 8-day embryos has no lethal effect, although a virus multiplication in the allantoic sac can be demonstrated. The 2-day embryos seem to be more sensitive than the 8-day embryos since an infection is invariably lethal in the former. Second, the incidence of malformations is low, and there is no indication of the occurrence of specific gross abnormalities. Unless the histological examination of the material should reveal lesions of inner organs the mumps virus must be considered as a lethal but not a teratogenetic agent.

Summary. Experimental evidence has been presented to show that influenza-A virus (PR8) has teratogenetic effects on the early chick embryo. It produces a specific syndrome, comprising microcephaly and micrencephaly, twist of the axis and impairment of the growth of the amnion. Furthermore, the virus is lethal for early embryos, within 3 days after infection. The mumps virus is likewise lethal for early embryos, within 5 days after infection. It does not produce specific abnormalities but seems to raise the incidence of malformations of the types which occur occasionally in uninfected chick embryos. These results place Influenza-A virus in line with rubella virus, as a teratogenetic agent. Furthermore, our observations on Influenza-A infections in chick embryos confirm the observations on rubella in humans in that only infections of early embryos result in abnormalities. Chick embryos of 4 days of incubation are killed by the influenza virus, but it seems that at this stage of development most organs have passed the critical period at which their morphogenesis can be directed into atypical channels. In this respect, it is

of interest to find that the patterns of infectiousness are different for the embryo and for the fully developed structures. In the embryo, the brain tissues seem to be particularly susceptible to Influenza-A virus, whereas in the adult the respiratory mucous membranes are primarily affected. In mumps, the infection of the salivary glands is not infrequently combined with meningitis, but no effect on the brain was found macroscopically in embryos. The situation is the same as in rubella where the embryonic defects seem to have no obvious relations to the manifestations of rubella infections in older phases of life.

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A Rapid Method for Demonstrating the Identity of Streptomycin-Producing Strains of *Streptomyces griseus*.*†

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In a search for new strains of the streptomycin-producing organism, *Streptomyces griseus*, it is usually necessary to make a large number of isolations from the soil or other natural substrates, test the ability of the organisms to inhibit the growth of various bacteria, grow them in liquid media favorable for the production of the antibiotic, isolate the latter from the medium, and establish its identity with streptomycin. Out of a hundred or more cultures of *S. griseus* thus isolated and tested,[1,2] only very few were found capable of producing streptomycin. Most of the cultures formed no antibiotic at all, whereas some produced other antibiotics, such as grisein.[3]

To overcome this difficulty, two procedures were adopted: 1. The use, for isolation purposes, of media containing streptomycin, so as to eliminate most of the bacteria and the great majority of actinomycetes that are sensitive to streptomycin. 2. The use of *S. griseus* actinophage for rapid spotting of the streptomycin-producing strains of *S. griseus*, since these were found[4] to be sensitive to this actinophage, whereas all other strains of *S. griseus* were resistant.

The results of the following experiment illustrate the use of these procedures. One gram samples of soil, cow manure, and compost materials were plated out, in dilutions of $1:10^4$, $1:10^5$, $1:10^6$, and $1:10^7$, with nutrient agar containing 25 μg of streptomycin per ml. The plates were incubated at 28°C for 5 days. Five actinomyces colonies were isolated from several of the plates and grown upon slants of glucose-asparagine agar. Good growth was obtained after 7 days' incubation.

The 5 cultures were streaked on nutrient agar plates and incubated for 24 hours; drops of actinophage, both undiluted and diluted 1:10, were placed upon some of the streaks. The plates were again incubated for 24 hours at 28°C, and examined; the streaks that were treated with actinophage showed inhibition of sporulation and lysis of the vegetative mycelium.

A duplicate set of plates was streaked with the freshly isolated cultures, incubated for 48 hours, and cross-streaked with four test bacteria.[5] The plates were incubated for an additional 48 hours, and the zones of bacterial

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1 Waksman, S. A., Schatz, A., and Reynolds, D. M., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 73.

2 Waksman, S. A., Reilly, H. C., and Johnstone, D. B., *J. Bact.*, 1946, **52**, 393.

3 Reynolds, D. M., Schatz, A., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 50.

4 Reilly, H. C., Harris, D. A., and Waksman, S. A., *J. Bact.*, 1947, **54**

TABLE I.
Zones of Inhibition of Test Bacteria by Freshly Isolated Strains of *S. griseus*, as Measured by Agar-streak Method.
Zone of inhibition measured in millimeters.

Strain No.	*Escherichia coli*	*Bacillus mycoides*	*Staphylococcus aureus*	*Bacillus subtilis*
1	20	20	18	26
2	23	23	19	28
3	20	20	17	27
4	22	22	20	26
5	20	22	17	23

TABLE II.
Production of Streptomycin by Freshly Isolated Strains and Stock Culture of *S. griseus*.
Streptomycin, μg/ml.

Strain No.	Days of incubation		
	3	5	7
1	30	120	93
2	42	63	60
3	55	132	128
4	40	54	54
5	43	108	96
3475	42	81	65

inhibition recorded. The results (Table I) show that all the cultures were able to inhibit the growth of both gram-positive and gram-negative bacteria, the type of spectra obtained being reminiscent of streptomycin.[6]

The 5 cultures were inoculated into flasks with meat extract-peptone-glucose medium commonly used for the production of streptomycin. For comparative purposes, an active streptomycin-producing strain of *S. griseus* (No. 3475) was also used. The flasks were

FIG. 1.
Use of actinophage for identifying streptomycin-producing *S. griseus* cultures.

placed in a shaking machine in the incubator at 28°C. The activity of the culture filtrates was determined after 3, 5 and 7 days incubation by the cup method against a streptomycin standard (Table II).

The results show that the freshly isolated cultures were all active producers of streptomycin. That the antibiotic formed by the unknown strains of *S. griseus* was streptomycin or at least a streptomycin-like substance is illustrated by the similarity in the antibiotic spectra of the different preparations.

To illustrate further the effect of phage upon the unknown strains, the cultures were streaked upon agar plates. These were incubated for 24 hours, metal rings placed upon a portion of the area of growth of the organisms, and a few drops of actinophage placed inside each ring. The plates were incubated another 24 hours, the rings removed, and the plates examined. The action of the phage is illustrated in Fig. 1. The effect of the phage was exactly the same as that usually produced on the streptomycin-producing strains of *S. griseus*.

Summary. A method for the rapid isolation and identification of streptomycin-producing strains of *Streptomyces griseus* is described. The method consists in plating out various dilutions of material from natural substrates, using agar media to which 25 μg/ml streptomycin has been added. Colonies of actinomycetes developing on the plates are picked and transferred to agar slants. When the cultures have developed, they are used to make agar streaks on plates. The plates are incubated for 24 hours, and drops of actinophage placed upon some of the streaks. The destruction of the growth of the actinomyces will prove the identity of the culture with streptomycin-producing *S. griseus*. This identification can be confirmed by the use of the agar-streak method, using various test bacteria, the sensitivity of which to streptomycin is known. Finally the cultures are grown in media used for the production of streptomycin, and the antibiotic produced is compared with streptomycin.

5 Waksman, S. A., and Reilly, H. C., *Anal. Ed., Ind. and Eng. Chem.*, 1945, **17**, 556.

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16174 P

Enhancement of Heterophile and Bacterial Agglutination Titers by Means of Serum Diluent.*

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Various workers have shown that Rh agglutination titers are enhanced, and the action of the so called "blocking" antibody inhibited by the use of serum, plasma or bovine albumin instead of saline as a diluent.[1-6] Recently Griffiths[7] has reported the inhibition of Brucella blocking antibodies when rabbit serum is used in place of saline as a diluent in agglutination tests. This paper is a preliminary report on the enhancement of heterophile and various bacterial agglutinin titers by use of serum diluent instead of physiological saline. Routine agglutination tests were done comparing saline and pooled human

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3 Diamond, L. K., and Denton, R. L., *J. Lab. and Clin. Med.*, 1945, **30**, 821.

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5 Levine, P., *Am. J. Clin. Path.*, 1946, **16**, 597.

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7 Griffitts, J. J., *Pub. Health Rep.*, 1947, **62**, 865.

or rabbit serum as a diluent. Titrations using plasma, ascitic fluid and 20% bovine albumin diluents were also carried out.

Methods. Heterophile agglutination tests were done according to the technic of Paul and Bunnell.[8] Serial twofold dilutions were made of the patient's serum in saline diluent, and equal parts of 1% sheep cells in saline were added to each tube. The tubes were shaken and incubated at 37°C for 2 hours. Next they were removed and kept overnight in the icebox at 5°C. The titer was read as the highest dilution showing distinct visible clumps of erythrocytes. The tests using human or rabbit serum diluent were carried out in an identical manner except that serum was used in place of saline. The serum diluent was inactivated by heating at 56°C for ½ hour and tested for the presence of heterophile antibodies prior to use. Only serum diluent having no heterophile agglutinins was used. The Davidsohn guinea pig kidney absorption test for infectious mononucleosis[9] was carried out in a similar manner with the patient's serum.

The bacterial agglutination tests were performed in the following manner: Serial twofold dilutions of the patient's serum previously inactivated by heating at 56°C were made both in saline and serum diluents having no agglutinins for the test organism. The serum diluent was heated at 56°C for ½ hour before use. Equal parts of various bacterial antigens suspended in saline and adjusted to a turbidity of nephelometer (McFarland) tube No. 3 were added to each tube. The tube mixtures were shaken and incubated at 56°C for 2 hours. They were then kept overnight in the icebox at 5°C and read the following morning. The following bacterial antigens were studied: typhoid, paratyphoid A and B, Shigella, Brucella and paracolon.

Results. Heterophile Agglutination. Typical heterophile agglutinin titrations comparing saline and serum diluents in individuals with positive and negative clinical and hematological† findings for infectious mononucleosis are summarized in Table I. Human plasma, ascitic fluid, rabbit serum, and 20% bovine albumin diluents also significantly enhanced heterophile agglutination titers. As shown in Table I heterophile agglutinins were detected in the serum of one patient (SK) with positive clinical and hematological findings for infectious mononucleosis using serum diluent, although repeatedly negative results were obtained in the routine saline test. In another positive case (EM) significant titers of heter-

TABLE I.
Enhancement of Heterophile Agglutination Titers by Means of Serum Diluent.

Patient	State of serum	Agglutinin titer	
		Saline diluent	Serum diluent
Positive Clinical and Hematological Findings.			
EN	Un*	1:5,120	1:40,960
	Abs†	1:1,280	1:5,120
EM	Un	1:80	1:1,280
	Abs	1:20	1:320
MC	Un	1:80	1:640
	Abs	1:20	1:160
SK	Un	0	1:80
	Abs	0	1:20
LM	Un	1:320	1:2,560
	Abs	1:80	1:320
DD	Un	1:40	1:640
	Abs	1:20	1:160
SR	Un	1:160	1:1,280
	Abs	1:40	1:160
IM	Un	1:5,120	1:40,960
	Abs	1:1,280	1:5,120
FS	Un	1:80	1:640
	Abs	1:40	1:160
Negative Clinical and Hematological Findings.			
WW	Un	1:20	1:320
	Abs	0	0
WA	Un	0	0
	Abs	0	0
WM	Un	1:10	1:80
	Abs	0	0
HM	Un	1:10	1:160
	Abs	0	0
JG	Un	0	0
	Abs	0	0
CR	Un	0	0
	Abs	0	0
GN	Un	0	0
	Abs	0	0
DR	Un	0	0
	Abs	0	0
MM	Un	0	1:160
	Abs	0	0
GW	Un	0	1:320
	Abs	0	0

* Unabsorbed.
† Absorbed with guinea pig kidney suspension.

8 Paul, J. R., and Bunnell, W. W., *Am. J. Med. Sciences*, 1932, **183**, 90.

9 Davidsohn, I., *J. A. M. A.*, 1937, **108**, 289.

† We are indebted to Dr. Karl Singer of the Hematology Department of Michael Reese Hospital for hematological studies.

TABLE II.
Enhancement of Bacterial Agglutination Titers by Means of Serum Diluent.

Patient	Test antigen	Agglutinin titer: Saline diluent	Agglutinin titer: Serum diluent
PN	Typhoid H	0	0
	" O	1:320	1:2,560
	Paratyphoid A	1:80	1:160
	" B	1:160	1:1,280
NL	Flexner V	1:80	1:640
	" Y	1:160	1:640
	Sonne	0	0
	Schmitz	1:80	1:640
	Shiga	0	0
JC	Flexner V	1:320	1:5,120
EG	*Brucella abortus*	1:40	1:160
	" *melitensis*	1:80	1:80
	" *suis*	1:40	1:80
JB	Typhoid O	0	0
	" H	1:320	1:1,280
	Paratyphoid A	0	0
	" B	1:160	1:1,280
CE	Paracolon	0	1:320

ophile agglutinins in both unabsorbed and absorbed serum were detected 5 days earlier with serum diluent. The confirmatory value of the guinea pig kidney absorption test in diagnosis of infectious mononucleosis[9] is emphasized by the fact that serum diluent failed to enhance titers of absorbed serums in negative cases.

Bacterial Agglutination. Preliminary results showing the enhancement of various bacterial agglutinin titers by means of serum diluent are given in Table II. It was noted that clumps of agglutinated bacteria in serum diluent were more easily dispersed on vigorous shaking than in saline. A similar enhancement of titer was observed with plasma, ascitic fluid, rabbit serum and 20% bovine albumin diluents. It is of interest that agglutinins for a paracolon bacillus isolated from a paratyphoid-like infection were demonstrable only by means of serum diluent.[10]

Comment. We feel that our results together with those previously reported by others suggest that not only does serum diluent inhibit the action of blocking antibody in certain serum, but that it is probably a more favorable medium in which the agglutination reaction can occur. Perhaps the presence of agglutinins can be detected sooner in the course of an infection by the routine use of serum diluent.

Summary. Heterophile and various bacterial agglutination titers were significantly enhanced by the use of serum diluent instead of saline. Similar results were obtained with plasma, ascitic fluid and bovine albumin diluents.

[10] Milzer, A., unpublished studies.

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Effect of Estrogen on Plasma Vitamin A of Normal and Thyroidectomized Rabbits.*

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The congenital malformation in the eyes of young rats produced by a severe maternal deficiency of vitamin A during pregnancy,[1,2] appears to have a strong resemblance to the fibroplasia which has been observed to occur spontaneously behind the lenses of some premature human infants.[3] Since it seems unlikely that this retrolental fibroplasia in humans is due directly to a maternal deprivation of vitamin A,[2] other influences which might cause

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[1] Warkany, J., and Schraffenberger, E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 49.

[2] Jackson, B., and Kinsey, V. E., *Am. J. Ophthalm.*, 1946, **29**, 1234.

[3] Terry, T. L., *Am. J. Ophthalm.*, 1942, **25**, 1409; *Arch. Ophthalm.*, 1943, **29**, 36, 54.

TABLE I.
Liver Vitamin A Level of Normal and Thyroidectomized Rabbits Treated with Large Doses of Estrogen.

	Thyroidectomized			Normal		
	No. of animals	Liver Vit. A I.U./g	Plasma Vit. A I.U. %	No. of animals	Liver Vit. A I.U./g	Plasma Vit. A I.U. %
Control	4	80	401	4	64	206
Estrogen treated	4	55	245	4	47	182

a general, or even a local, deprivation of vitamin A were sought.

During the course of pregnancy, the blood vitamin A level falls markedly and continuously so that an abnormally low level obtains at term. This low vitamin A level returns to approximately normal in about 24 hours after parturition.[4,5] The behavior of the blood vitamin A during pregnancy appears to be the converse of that reported for the blood and urine estrogen levels; the estrogen output increases to a very high level and then drops precipitously directly after parturition.[6,7] An earlier report has also suggested that estrogens may inhibit the growth-stimulating effect of carotene.[8]

It was considered to be of value, therefore, to establish whether a relationship exists between the administration of estrogens and the blood level of vitamin A. Since thyroid material has a marked influence on both of the above substances,[9,10] the role of the thyroid has been taken into account in this study.

Experimental. Young animals were used in order to reduce the effect of their own sex hormones to a minimum. Weanling, albino rabbits were maintained on a normal chow diet. The rabbits in Exp. 1 were all females from one litter and were thyroidectomized at 5½ weeks of age.

Two weeks after thyroidectomy, the intramuscular injection of estrogen (α estradiol benzoate in peanut oil†) was begun. An injection of 2,000 R.U. (0.33 mg) was given twice a day to each animal; one day a week only one dose was injected. Thus, 64,000 R.U. were given to each experimental animal over 17 days in Exp. 1, and 68,000 R.U. over 19 days in Exp. II. The animals used in the second experiment were not thyroidectomized but were started on the estrogen treatment when approximately 6 weeks old. These rabbits were from 2 litters, 4 being females, and were distributed equally between the "control" and "estrogen treated" groups.

A blood sample was obtained by heart puncture before the first injection of estradiol and at various intervals during the course of the experiment. Finally, the animals were killed and the livers removed. The plasma samples were analyzed for their vitamin A content by the micromethod of Bessey, *et al.*[11] Aliquots of the livers were digested in 5-10 volumes of alcoholic KOH (5N KOH in 60% ethanol) on a hot water bath for 30 minutes and were then extracted with a 1:1 mixture of xylene and kerosene. The vitamin A concentration was then determined by the same procedure as that used for the determination in the plasma.

[4] Jackson, B., and Kinsey, V. E., private communication.

[5] Goldberger, M. A., and Frank, R. T., *Am. J. Obstet. Gynecol.*, 1942, **43**, 865.

[6] Cole, H. H., and Saunders, F. J., *Endocrinology*, 1935, **19**, 199; Smith, G., Smith, O. W., and Pincus, G., *Am. J. Physiol.*, 1938, **121**, 98; Cohen, S. L., Marrian, G. F., and Watson, M. C., *Lancet*, 1935, **228**, 674.

[7] Lund, C. J., and Kimble, M. S., *Am. J. Obstet. Gynecol.*, 1943, **46**, 486.

[8] von Euler, H., and Klussmann, E., *Arkiv Kemie, Mineral Geol.*, 1932, **11**B, No. 1; *C. A.*, 1934, **26**, 3827.

[9] McDonald, M. R., Riddle, O., and Smith, G. C., *Endocrinology*, 1945, **37**, 23.

[10] Greaves, J. D., and Smith, C. L. A., *Am. J. Physiol.*, 1936, **116**, 456.

[11] Bessey, O. A., Lowry, O. H., Brock, M. J., and Lopez, J. A., *J. Biol. Chem.*, 1946, **166**, 177.

† Part of the estradiol benzoate used was kindly furnished by Dr. F. E. Houghton of the Ciba Pharmaceutical Products, Inc.

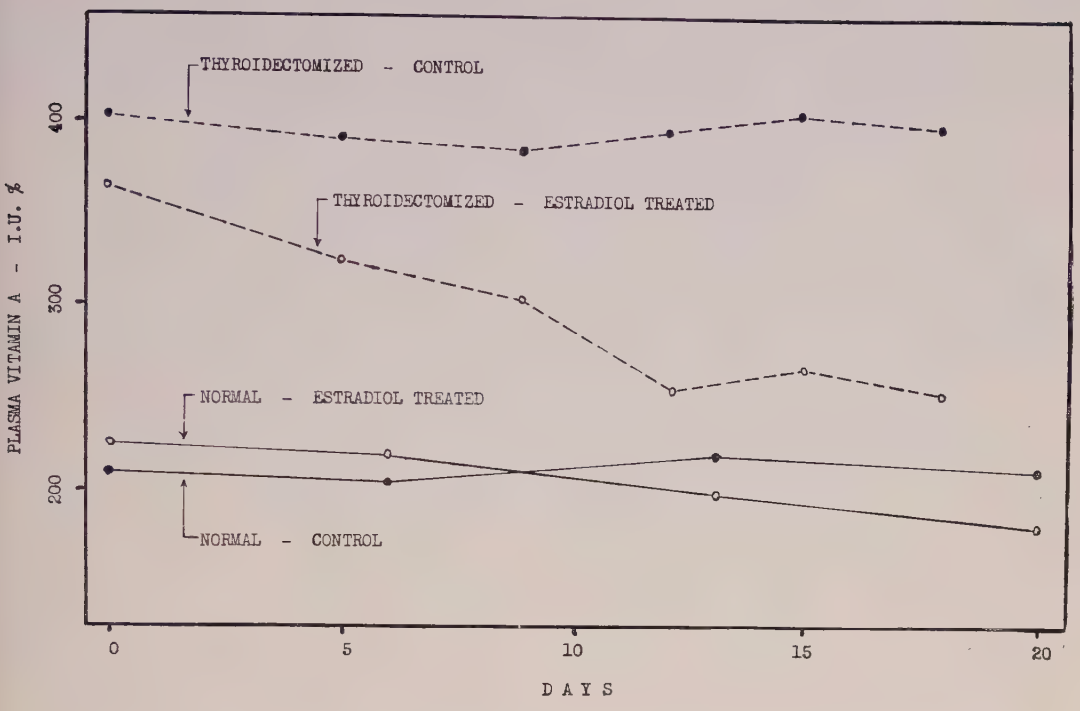

FIG. 1.

Plasma vitamin A as affected by the administration of α-estradiol benzoate. Broken line shows results obtained from thyroidectomized rabbits; solid line, from non-thyroidectomized normal animals.

The results of these experiments are shown in Fig. 1. The plasma vitamin A remained essentially unchanged in the control animals of both experiments. However, the level of vitamin A in the plasma of the estrogen treated rabbits showed a decline, particularly in the case of the thyroidectomized ones. The decrease observed in the non-thyroidectomized rabbits does not appear to be significant. It was found that the plasma level of vitamin A was higher in the thyroidectomized animals than in the normals. This higher level is probably due to the decreased metabolism of vitamin A observed after removal of the thyroid. The vitamin A content of the livers reflects the final plasma level determinations (Table I).

A possible explanation of the observed decrease in vitamin A may be found in the experiments of Loeb.[12] He reports that the stores of body fat are increased under the influence of repeated estrogen injections. It would appear likely that the fat which is deposited may carry along some vitamin A, thereby lowering the plasma level.

The amount of estrogen used in these experiments was much greater than might be expected to be secreted by the pregnant animal. Yet, this large amount of estrogen did not depress the plasma vitamin A level below normal. It would then seem doubtful that the estrogens normally produced during pregnancy could influence the metabolism of vitamin A sufficiently to be considered a cause of the fibroplasia found in the eyes of premature infants.

Summary. Thyroidectomized and normal young rabbits were injected with large doses of estradiol benzoate for 17-19 days. The vitamin A level in the plasma of the thyroidectomized animals was significantly lowered. There was no appreciable decrease in the plasma vitamin A of the normal rabbits.

12 Loeb, H. G., PROC. SOC. EXP. BIOL. MED., 1942, **51**, 330.

16176

Thyroid Hyperplasia in Rats Following Thymus Feeding.

K. Kjerulf-Jensen. (Introduced by H. Dam.)

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Hou[1] stated that rats fed on a soybean-millet diet with dried liver powder as the only source of animal protein developed a marked hypertrophy of the thyroid gland.* The thyroid hyperplasia did not seem to be due to administration of the animal protein as such, since rats given dried beef powder instead of liver powder developed only a relatively slight hyperplasia. The goitrogenic principle could be removed from the liver tissue by alcohol extraction. This pronounced goitrogenic effect of dried liver powder in Hou's experiments was so considerable that, according to our experience, feeding with about 0.1 mg 2-thio-4-methyl-uracil per 10 g diet would be required to produce a comparable enlargement of the thyroid gland.

The goitrogenic effect observed in the experiments reported below was not so pronounced as that seen in Hou's experiments. On the other hand, thyroid hyperplasia could be elicited not only by adding dried thymus powder to the soybean-millet diet, but also by giving a usual standard diet with addition of thymus. It was impossible in this case to remove the goitrogenic substance in the thymus tissue by alcohol extraction. Addition of corresponding amounts of dried ox-liver powder to the soybean-millet diet caused very little thyroid hyperplasia in these experiments. Dried beef powder had no goitrogenic effect. The goitrogenic effect of the thymus administered did not seem to be due to the contents of nucleoprotein as such, since no goiter was developed after administration of dried cod spawn in corresponding amounts.

Groups of 5 rats each were given the different diets. (The initial weights of the animals ranged from 60 to 120 g, so that the body weights by the end of the experimental periods differed but little). The rats were killed after 30, 60, and 90 days respectively, and their thyroids were excised, weighed, and submitted to histological examination. The weights were indicated in percent of the normal, as pointed out in a previous paper by Jensen and Kjerulf-Jensen.[2]

The diet consisted of the following substances:

Soybean flour	450
Millet	500
Sodium chloride	20
Cod liver oil	1
Ascorbic acid	0.5

This diet was supplemented by

1. Dried thymus of young calves: 30 (1 g dried thymus corresponded to 4.7 g fresh thymus).
2. Residue of dried thymus gland, extracted by 5 vol. of ethyl alcohol at 25°C, in the above amounts.
3. Water-soluble fraction of the alcohol extract of dried thymus in the above amounts, as well as dried beef: 30.
4. Water-insoluble fraction of alcohol extract of dried thymus in the above amounts, as well as dried beef: 30.
5. Dried beef powder: 30.
6. Dried ox liver powder: 30.
7. Alcohol extract of dried ox liver in the above amounts, as well as dried beef: 30.
8. Dried cod spawn: 30.

The histological conditions were indicated as: 0 = normal, or: + = slight, *i.e.* slight cellular hyperplasia with increase in the height of the cells of the glandular epithelium, and inconsiderable reduction of the amount of colloid substance, or: ++, *i.e.* increase in cell height as well as marked reduction of the amount of colloid substance.

Feeding with dried thymus elicited thyroid hyperplasia also in rats given the usual laboratory diet instead of the soybean-millet diet mentioned above. Administration of up to 10 times as much thymus added but little

[1] Hou, C. H., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 753.

* For further references to literature *vide* the quoted article by Hou.

[2] Jensen, K. A., and Kjerulf-Jensen, K., *Acta pharmacol.*, 1945, **1**, 280.

TABLE I.
Thyroid Hyperplasia of Rats after 1, 2, or 3 Months of Feeding with Soy Bean-Millet Diet Supplemented by Dried Thymus, Beef, Ox Liver, or Cod Spawn Respectively

Diet No.	Avg size of thyroid in % of norm. for groups 5 rats for 30, 60, and 90 days respectively			Cellular hyperplasia of thyroid, estimated for each individual group as an entirety		
1. Dried thymus	123	130	126	+	++	++
2. Thymus, extr. by alcohol	120	124	136	++	++	++
3. Water soluble fract. of thymus alc. extr.	143	108	130	+	0	+
4. Water insolub. fract. of thymus alc. extr.	99	140	138	0	+	+
5. Dried beef	117	144	124	0	0	0
6. Dried liver	137	116	113	0	0	+
7. Alcohol extr. of ox liver	111	153	123	0	+	+
8. Dried cod spawn	115	106	108	0	0	0

to the increase in the size of the thyroid, whereas the histological changes, *i.e.* the cellular hyperplasia and especially the reduction of colloid substance became particularly pronounced.

Summary. Moderate cellular hyperplasia of the thyroid was produced in rats on a soy-bean-millet diet or a usual standard diet by adding dried thymus from calves.

The goitrogenic fraction was insoluble in water and ethyl alcohol; no goitrogenic effect was observed after feeding with dried ox liver, dried beef or dried cod spawn.

16177

Isolation and Properties of Raphanin, an Antibacterial Substance from Radish Seed.

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Antibacterial substances are not rare in various higher plant species.[1] In the course of a systematic investigation of aqueous extracts of plants belonging to the family of Cruciferae for the presence of antibiotic principles we found[2] that extracts of the seeds of radish (*Raphanus sativus*), when tested by the cylinder plate method (Heatley[3]), gave a wide zone of inhibition on plates seeded either with *Staph. aureus* or with *B. coli.* The substance responsible for the action could be purified and finally isolated as a homomolecular liquid.

Experimental. The aqueous extract of the seeds is very effective in preventing the growth of bacteria. The cell-free extract prepared by the extraction of one part of finely ground seeds with 5 parts of water gave a 30 mm zone of inhibition on plates seeded with *Staph. aureus* or *B. coli.* Even extracts prepared in the ratio of 1:50 caused marked inhibition. The antibacterial principle is fairly resistant to heat; only a slight decrease in potency was observed after heating it to the boiling point for 30 min. The cell-free extract will retain full activity for weeks if stored in the ice box. However, all activity is lost within 24 hours if the extract is incubated with the crushed seeds at 37°C. Considerable inactivation occurs under such conditions even at 20°C; in fact, even at ice box temperature. On the other hand, heating the suspension to 60°C for 15 min. completely prevents inactivation.

1 Kavanagh, F., *Adv. Enzymology*, 1947, **7**, 461.
2 Ivánovics, G., and Horváth, S., *Nature*, 1947, **160**, 297.
3 Heatley, N. G., *Bioch. J.*, 1944, **38**, 61.

TABLE I.
Extraction of Antibiotic by Various Solvents from Aqueous Extract of Seeds.

Solvent	pH of extract	Diameter of zone of inhibition in mm: Dilution of organic solvent fraction 1:1	1:2	1:3	Dilution of aqueous residue 1:4	1:8	1:12
Amyl acetate	2	24	22	21	22	16	15
" "	3	24	22	20	20	17	15
" "	4	26	24	19	21	17	14
Butanol	3	27	26	26	16	14	8
Ethyl acetate	3	28	26	25	22	16	13
Petroleum ether	3	13	9	0	27	22	20

If intact or carefully peeled seeds are soaked in water for a few hours the diffusate is found to be devoid of activity, thus indicating that the active principle is firmly bound to the cells. Since the active substance obtained from crushed seeds passes a cellophane membrane with ease it is obvious that the seeds do not contain the active principle proper. This fact, together with the heat resistance of the active extract and with the failure to obtain active extracts from seeds boiled 15 min. before crushing them, led to the assumption that the seeds contain an enzyme capable of converting an inactive precursor into an antibiotic. The correctness of this assumption could be proven by the following experiment: Crushed seeds were extracted with 3 changes of 80% ethanol; the extract was concentrated and freed from alcohol *in vacuo* (extract A). Another portion of the crushed seeds was extracted with water and dialyzed in a cellophane bag against tap water (extract B). Both extracts were entirely devoid of any antibacterial activity. However, when the extracts were mixed, and the mixture allowed to stand for one hour, a potent solution resulted. No activity was obtained if extract B was heated to 70°C for 30 min. before adding it to extract A.

Crude extracts of the active principle were prepared as follows: 2000 g of finely ground seeds was stirred up with 3000 ml of tap water, and the mixture allowed to stand at room temperature for 30 to 40 min. The suspension was strained through cheesecloth, and the residue squeezed out in a hand press. The combined filtrates were adjusted to pH 5, and 14 to 15 ml of a 40% solution of basic lead acetate was added to each 100 ml of the juice. The precipitate was centrifuged off and discarded. The excess lead, which otherwise would have caused gradual inactivation, was removed by the addition of 7 to 8 ml of a 20% solution of $Na_2HPO_4 \cdot 12H_2O$. The supernatant gave only a very slight reaction with H_2S. It should be remarked here that the excess lead must not be removed with H_2S to which the active principle is sensitive. This procedure of purification caused a loss not exceeding 20%. The extract thus obtained will be referred to as the purified aqueous extract.

The active principle can be extracted with ease by various organic solvents at an acid pH. 40 ml of the purified aqueous extract was shaken with 20 ml of various organic solvents. The solvents were subsequently evaporated *in vacuo*, and the residue redissolved in 10 ml of water. Both the original aqueous phase and this latter solution were assayed by the cylinder plate method. The results are shown in Table I where the concentration of the redissolved organic solvent fraction is referred to as 1:1, while that of the original aqueous phase is taken as 1:4, in comparison to the other fraction.

The active principle is not absorbed on tricalcium phosphate or on kaolin. However, it is easily adsorbed on charcoal at pH 5 to 7. All attempts at elution between pH 2.2 and 11 failed.

In further experiments a butanol concentrate of the active principle served as a standard of reference. An amount producing a 19 to 21 mm wide zone of inhibition was chosen as an arbitrary unit of activity.

The pure active principle was isolated in

TABLE II
Elementary Composition of Raphanin and of Inactive Crystalline Substance.

Solvent used for extraction	Substance	% composition							
		C		H		N		S	
Butanol	Raphanin, Batch 3								
	First fraction	41.71		5.03					
		41.10	41.27*	5.41	5.34*				
		41.00		5.58					
	Middle "	41.23	41.26*	5.28	5.18*	8.41			
		41.30		5.08					
	End "	41.66		5.27					
Butyl acetate	Raphanin, Batch 5†	41.62	41.50*	5.61	5.50*	8.91		33.94	33.64*
		41.38		5.40				33.34	
	Inact. cryst. substance	34.64		5.48		6.89		45.42	45.36*
		34.71	34.65*	5.09	5.31*	6.70	6.73	45.31	
		34.60		5.35		6.60			
Theoretical values for	$C_{17}H_{26}N_3O_3S_5$	42.48		5.45		8.74		33.35	
	$C_{17}H_{26}N_3O_4S_5$	42.11		5.28		8.58		33.03	

* Average values
† Freshly redistilled by molecular distillation.

the following manner: 5330 ml of the purified aqueous extract containing 7370 units of the antibiotic was obtained from 2000 g of seeds. This solution was extracted with six 1000 ml portions of butyl acetate. The extracts were combined and assayed; total recovery was 5648 units. The solvent was removed by vacuum distillation. The resulting brown oily material was shaken with 200 ml of 0.07M phosphate buffer pH 7.2; the insoluble material was filtered off and discarded. The water-soluble fraction was extracted with 3 portions of chloroform, 30 ml each. The combined chloroform extracts contained 5500 units. The solution of the antibiotic in chloroform was shaken with 100 ml of phosphate buffer to remove the last traces of acidic impurities. The active principle, together with most of the pigment, remained in the chloroform layer. The solution was now passed through a column of Brockman's alumina which adsorbed a brick-red pigment. The percolate contained all the activity. After evaporation of the chloroform and of the last traces of moisture, a slightly yellow liquid of syrupy consistency was obtained. It had a sharp boiling point and could be distilled under reduced pressure without much decomposition. Boiling point: 135°C at 0.06 mm; 142.5°C at 0.09 mm, and 145°C at 0.1 mm Hg. Total yield, 7.5 g.

The distillate which consisted of the pure active principle was designated as raphanin. The homogeneity of the distillate could be established by redistillation at 135°C under a pressure of 0.06 mm Hg. Fractions were collected separately at intervals at the beginning, the middle and the end of the procedure and analyzed for C, H, N and S (Table II).

Raphanin is only moderately soluble in ether. The partition coefficient between butanol and water is favorable but butanol extracts contain a large amount of impurities which can be removed only with difficulty.

In an effort to reduce the amount of solvent required for extraction, the purified aqueous extract was concentrated *in vacuo* at 60°C to about one-half of its original volume. In one experiment 4500 ml of purified aqueous extract was reduced to 2000 ml. This operation resulted in the loss of about 1000 units. The concentrated liquid was extracted with butyl acetate; the solvent was evaporated *in vacuo* to a small volume. This concentrate was placed in the refrigerator, and after standing there overnight, a crop of crystals appeared. The crystals were collected, recrystallized first from boiling water, then from ethanol. Yield, 400 mg M. P. 192°C (decomp.). The elementary analysis is shown in Table II. This crystalline substance did not possess any antibacterial activity.

Some physical and chemical properties of raphanin. Freshly distilled raphanin has a

TABLE III.
Highest Dilutions of Raphanin Causing Inhibition of Bacterial Growth.

Organism	Medium	Partial inhibition × 1000	Complete inhibition × 1000
Staph. aureus	broth	1:50	1:28
" "	serum broth*	1:1	1:1
" "	casein hydr. broth†	1:50	1:28
B. coli	broth	1:10	1:5
" "	serum broth*	1:1	1:1
" "	synthetic‡	1:125	1:70
Salm. schottmülleri	broth	1:8	1:5
B. anthracis	"	1:8	1:4
Pseud. aeruginosa	"	1:16	1:8
" "	serum broth*	1:4	1:2
Salm. typhi	broth	1:16	1:8
" "	serum broth*	1:4	1:2

* Containing 50% ox serum.
† According to Ivánovics, *Acta Med. Szeged*, 1944, **12**, fasc. 1.
‡ According to Sahyun, Beard, Schultz, Snow, and Cross, *J. Infect. Dis.*, 1936, **58**, 58.

TABLE IV.
Zones of Inhibition in mm with Various Concentrations of Raphanin.

Organism	Zone of inhibition in mm		
	1 mg/ml	2 mg/ml	3 mg/ml
Staph. aureus	18.5	22.5	24
B. anthracis (avirulent)	23	26.5	28
" " (virulent)	22	26.5	29
" *subtilis* (strain Duthie)	20	26	29
" *coli*	16	19.5	21
Salm. typhi—H	19	24.5	27
" " —O	24	26.5	28.5
" *schottmülleri*	22	28.5	31
B. shiga-kruse	16	20	22
Pseud. aeruginosa	19	23	25
B. prodigiosus	12	15	19

slightly yellowish shade which will darken on storage at room temperature. It was attempted to obtain a colorless sample by molecular distillation at 60°C. The distillate had a very minimal yellowish tinge; it was impossible to secure an entirely colorless preparation. It is believed that the yellowish shade is due to decomposition products although darkening at room temperature is not associated with any noticeable loss in antibacterial activity.

The substance has a radish-like odor. It is quite soluble in water with a neutral reaction. Its solution in absolute ethanol is levorotatory; $[\alpha]_D^{20} = -141°$. Freshly prepared aqueous solutions give no coloration with ferric chloride or with nitroprusside. However, after treatment with dilute HCl and Zn dust, an intense purple shade was obtained with nitroprusside, indicating the presence of –SH groups. If the aqueous solution is heated with Ag or Pb salts, a heavy black precipitate, insoluble in HNO_3, is obtained, and the supernatant is found to have lost its antibacterial activity.

If raphanin is shaken with 0.1N $Ba(OH)_2$ at room temperature, the excess alkali neutralized with H_2SO_4, the $BaSO_4$ precipitate removed by filtration, and the filtrate, evaporated to a small volume, is placed in the refrigerator, crystals will precipitate on standing. This crystalline substance is inactive. M.P., after recrystallization from ethanol, is 192-93°C (decomp.). When these crystals were mixed with those obtained from the butyl acetate concentrate, no depression of the melting point was observed.

Antibacterial activity of raphanin. Raphanin was assayed on a number of bacterial species, using both the serial dilution method

TABLE V.
Residual Antibacterial Activity of Raphanin After Heating Its Solutions for 30 min. at Differenut pH (expressed in per cents of original activity).

Buffer	pH	Temperature 20	60	80	100°C
Phosphate	5.3	100	100	85	40
,,	7.2	100	80	50	0
Glycine	8.0	90	75	50	0
,,	10	50	0	0	
,,	11	40	0	0	

and the cylinder plate technique. An aqueous solution of freshly distilled raphanin was sterilized by filtration through a fritted glass plate. Various amounts of raphanin were added to 4 ml of nutrient medium, and the volume was made up with dist. water to 5 ml. The tubes were inoculated with 0.1 ml of a dilute bacterium suspension containing about 50,000 organisms in one ml. They were incubated for 24 hours at 37°C. (Table III). In tests by the cylinder plate method 3 different dilutions containing 1, 2 and 3 mg of raphanin per ml were used (Table IV).

The activity of raphanin against *B. coli* is superior to that of crystalline penicillin G since a 1:1000 dilution of raphanin gave a zone of inhibition of 18 mm, whereas penicillin in the same dilution gave a zone of only 13 mm. Even a 1:5000 dilution of raphanin produced noticeable inhibition while penicillin in a strength of 1:3000 was without effect.

Raphanin is readily inactivated in the alkaline range while it is much more stable at a neutral or slightly acid solution. 25 mg of raphanin was dissolved in 5 ml of buffer solution; the solutions were incubated at various temperatures for 30 min, and the activity was tested by the cylinder-plate method (Table V).

As mentioned, raphanin is rapidly inactivated by H_2S. However, this activation is dependent on the pH of the medium. If H_2S is bubbled through a buffered solution of raphanin for 3 min, and the gas subsequently removed by aeration, complete inactivation occurs at pH 7.2, but only 50% inactivation at pH 5.

Toxicity of raphanin. Owing to an acute shortage of experimental animals, the toxicity of raphanin could be tested only in a small number of mice and guinea pigs. Mice weighing 16 to 22 g were injected i.v. with doses of raphanin ranging from 5 to 40 mg. Five mg caused only transitory weakness, excitement and ruffled fur, and the animals recovered promptly. Doses of 7 to 10 mg killed the animals within 5 to 10 min, while doses of 20 to 40 mg caused death in a few seconds. The results were very similar when raphanin was injected subcutaneously, except that the appearance of the symptoms was somewhat delayed. The symptoms after the injection of a lethal dose were weakness, restlessness, dyspnea and clonic convulsions. Animals injected with 10 to 20 mg scratched their noses furiously with their hind legs. At post-mortem examination no gross anatomical changes were found, except for a slight edema at the site of injection.

Three guinea pigs, weighing 450 to 470 g, were injected intracardially with 25, 50 and 125 mg. No ill effect was observed after 25 mg; 50 mg caused death after 30 min, while the injection of 125 mg was followed by signs of weakness and excitement, and the animal died in convulsions after a few minutes.

In tissue cultures of rabbit testis, a 20,000 dilution of raphanin completely prevented the growth of fibroblasts; there was a definite inhibition of growth at a dilution of 1:40,000, while a dilution of 1:80,000 did not cause any inhibition.

Raphanin is a potent inhibitor of the germination of various plant seeds, including those of radish itself. Seeds were placed on discs of filter paper soaked in dilute solutions of the antibiotic. The discs were kept in a moist chamber for 3 days at room temperature. Results are shown in Table VI.

TABLE VI.
The Germination of Seeds of Different Plants in the Presence of Raphanin.

Plant species	Conc. of raphanin	No. of seeds	No. of seeds germinated	Avg length of roots in mm
Raphanus sativus	0 (control)	25	23	21.5
" "	1:200	20	1	3.0
" "	1:1,000	25	24	14.0
" "	1:10,000	25	25	20.0
Brassica oleracea	0	25	20	17.2
" "	1:1,000	25	8	4.0
" "	1:10,000	25	20	11.0
Festuca pratensis	0	20	14	8.6
" "	1:1,000	20	1	4.0
" "	1:10,000	20	13	5.3
Sinapis alba	0	25	19	10.2
" "	1:1,000	25	0	0
" "	1:10,000	25	12	12.5
Hordeum distichon	0	20	20	16.0
" "	1:1,000	20	6	7.7
" "	1:10,000	20	20	12.8

Comments. The molecular weight of raphanin has not been estimated yet but on the basis of data of the elementary analysis the empirical formula could be either $C_{17}H_{26}O_3N_3S_5$ or $C_{17}H_{26}O_4N_3S_5$. The negative nitroprusside reaction indicates the absence of –SH groups and rules out the existence of an unsaturated lactone ring in the molecule. On the other hand, the appearance of –SH groups after reduction with nascent hydrogen is suggestive of a disulfide linkage.

The seeds of some of the Cruciferae contain thioglucosides which are hydrolyzed by concomitant enzymes into sugars and aglycons, the latter being mustard oils (isothiocyanic esters). This fact is of importance because it is asserted[4] that allyl isothiocyanate and related mustard oils possess an antibacterial action, a statement not borne out by other observations.[5] We were also unable to demonstrate any antibiotic effect in the seeds of *Sinapis alba*. Raphanin, as can be judged from its composition and chemical behavior, does not seem to belong in the group of mustard oils, although data so far obtained do not permit any definite conclusions as to its exact structure. It bears some resemblance to gliotoxin isolated from *Aspergillus fumigatus* and to allicin. Both of these latter antibiotics contain a high percentage of sulfur in a disulfide linkage; both are effective against Gram-positive as well as against Gram-negative organisms, and both are rather toxic. It is interesting to note that the intact cells of garlic do not contain allicin but only an inactive precursor which is transformed by a concomitant enzyme into active allicin.[6]

Summary. The preparation and purification of a new antibiotic, raphanin, from the seeds of the radish is described. The seeds contain an inactive precursor which is activated to a potent antibiotic by a concomitant enzyme.

The physical, chemical, antibiotic and toxic properties of raphanin are described. Raphanin, owing to its high toxicity, does not hold the promise of a useful therapeutic agent.

[4] Foter, M. J., and Golick, A. M., *Food Res.*, 1938, **3**, 609.

[5] Osborn, E. M., *Brit. J. Exp. Path.*, 1943, **24**, 227.

[6] Cavallito, C. J., Bailey, J. H., and Kircher, F. K., *J. Am. Chem. Soc.*, 1944, **66**, 1950.

16178

Chronic Riboflavin Deficiency in the Rat. I. Ossification in the Proximal Tibial Epiphysis.*

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The importance of riboflavin in skeletal development has been recently emphasized by Warkany.[1] Warkany found that prenatal riboflavin deficiency in the rat resulted in the production of young with multiple skeletal abnormalities. Among the abnormalities were developmental disturbances of the mandible, shortening and distortion of the extremities, various forms of syndactyly of the hands and feet, and fusion of the ribs and sternal centers of ossification. Histologically, a more or less marked delay in ossification occurred with persistence of cartilage in many areas. Levy and Silberberg[2] recently reported that in the mouse deficiency of riboflavin from weaning resulted in retardation of endochondral ossification and increased "degeneration of cartilage."

In the present study the changes in endochondral ossification in the tibia that occurred in rats born from riboflavin-deficient mothers and maintaind for three to nine months on purified diets deficient in riboflavin are reported. The riboflavin deficiency produced under these experimental conditons was chronic in nature.

Experimental. Normal female rats of the Long-Evans strain, 2 to 3 months of age, were bred with normal males and placed on riboflavin-deficient or control diets the day of breeding. At birth the riboflavin-deficient young were carefully examined for skeletal abormalities. All litters† were weighed every 5 days, weaned on the twenty-first day and continued on the same diet as their parents.

Previous studies[3,4,5] have disclosed that riboflavin deficiency is accentuated by a high fat diet. For this reason 2 riboflavin-deficient diets were used; one was high in carbohydrate and the other, high in fat. The latter was given from 90 days of age to the end of the experiment. The high-carbohydrate diet was composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco), and 4% salts No. 4.[6] The high-fat diet contained 48% instead of 8% hydrogenated cottonseed oil with a corresponding reduction in the proportion of sucrose. Both diets contained the following crystalline B vitamins per kilogram of diet: 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 50 mg calcium pantothenate, 400 mg inositol, and one gram choline chloride. A fat-soluble vitamin mixture was given weekly and contained 325 mg corn oil (Mazola), 400 U.S.P. units vitamin A, 58 Chick Units vitamin D, and 3 mg alpha-tocopherol. During the lactation period the lactating mothers received a double

* Aided by grants from the Board of Research and the Department of Agriculture of the University of California, the Rockefeller Foundation, New York City, the American Foundation for Dental Science, and the California State Dental Association. Crystalline B vitamins and synthetic alpha-tocopherol were generously furnished by Merck and Co., Rahway, N.J.

1 Warkany, J., *Vitamins and Hormones,* 1945, **3**, 73, Academic Press, Inc., New York, Publishers.

2 Levy, B. M., and Silberberg, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 355.

† Six young were kept per litter.

3 Mannering, G. J., Lipton, M. A., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 100.

4 Mannering, G. J., Orsini, D., and Elvehjem, C. A., *J. Nutrition,* 1944, **28**, 141.

5 Czackes, J. W., and Guggenheim, K., *J. Biol. Chem.,* 1946, **162**, 267.

6 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.,* 1941, **138**, 459.

TABLE I.
Riboflavin Deficiency in Male Rats from Birth.

Group	Animal No.	At autopsy: Age (day)	At autopsy: Wt (g)	Length of tibia* (mm)		Width of proximal epiphyseal cartilage of tibia† (μ)	
I	B8492	96	317	39.2		244	
Riboflavin control	BH8494	110	352	41.2		165	
group	BH8487	144	436	38.2	Avg 40.5	163	Avg 143
	B7985	194	537	41.8		130	
	GH7980	239	520	39.6		144	
	BH7986	278	630	42.9		110	
II	B6491	96	41	24.1		124	
Riboflavin deficient	GH2467	110	94	32.7		114	
group	B2498	144	122	35.0	Avg 34.3	114	Avg 111
	BH2499	194	169	37.9		123	
	B2495	237	127	36.9		100	
	B2493	277	188	39.3		89	

* The length of the tibia was measured with calipers before decalcification.
† As measured in the central portion of histologic sections with an ocular micrometer.

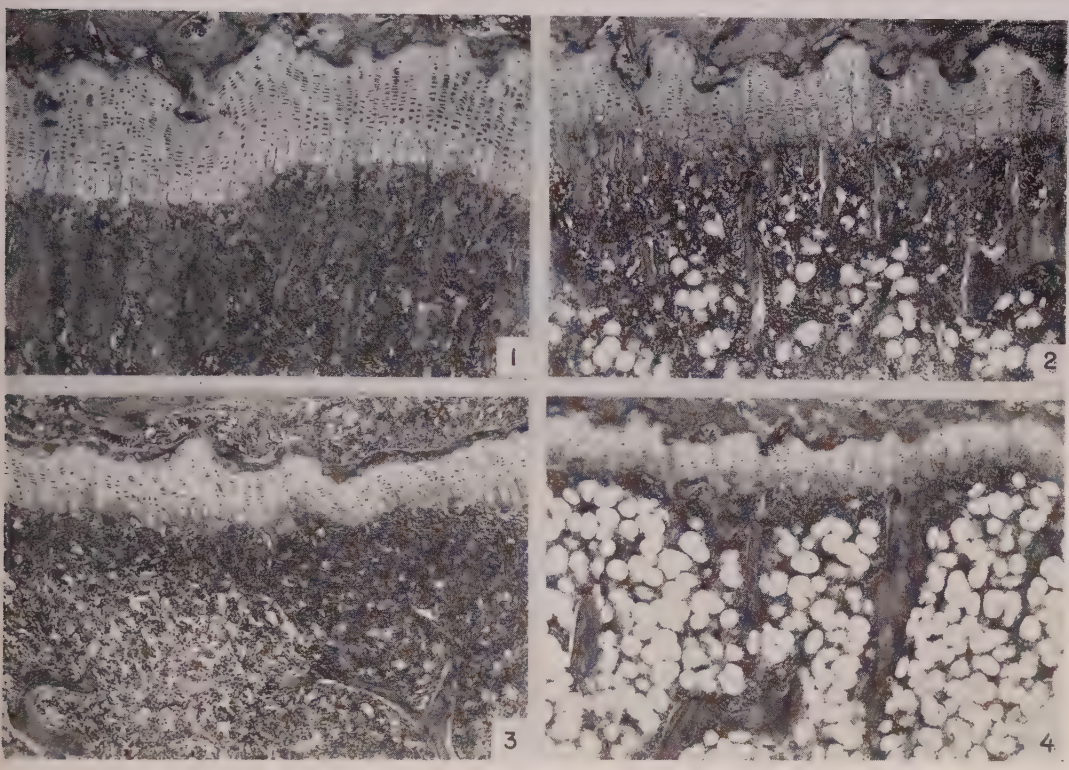
amount of the fat-soluble vitamin allotment. Control rats received the identical purified diet supplemented with 10 mg riboflavin per kilogram of diet.

For the histologic study of the tibia 6 riboflavin-deficient animals that were most severely affected as judged by external appearance and retardation of body weight were selected. These animals together with the riboflavin controls were autopsied at ages varying from 3 to 9 months, *i.e.* 96, 110, 144, 194, 237 and 278 days (Table I). The tibias were fixed in 10% neutral formol, roentgenographed and measured, decalcified in 5% nitric acid, embedded in nitrocellulose, sectioned at 8-10 micra, and stained with hematoxylin and eosin.

Results. During the first 90 days of the experiment on the high-carbohydrate diet, only a mild riboflavin deficiency was produced, the rats showing few deficiency symptoms other than retardation of growth. In agreement with the findings of previous investigators, the deficiency was accentuated by the high-fat diet. Dermatitis, alopecia, cataracts, spasticity, and marked decreases in growth occurred in many animals. However, the prolonged survival of many animals (see autopsy ages, Table I) showed that the deficiency was chronic rather than acute even with the high-fat diet. It may be mentioned that no skeletal abnormalities were observed in the young born from riboflavin-deficient mothers given either the high-carbohydrate or high-fat diets the day of breeding. Neither were abnormalities found in the second litters produced by these same mothers after they had received the deficient diets for 50-60 days at the time of the second breeding.

In comparing the tibial length and width of the epiphyseal discs marked differences were found between the riboflavin-deficient and the control group (Table I). The average length in the riboflavin-deficient group was 34.4 mm against 40.5 mm in the riboflavin-control group. Epiphyseal disc measurements averaged 111 micra for the deficient as compared with 143 micra for the control animals. Thus the decrease in epiphyseal disc width was aligned with shorter tibial length. The greatest changes in tibial length and cartilage disc width occurred in the youngest deficient animals. Severe decreases in body weight were found simultaneously (Table I).

The histologic aspect of the epiphyseal cartilage of the tibia in normal rats and changes occurring with increasing age have been recently presented in detail.[7] In this study measurements of tibial length and epiphyseal disc width of the riboflavin control animals were slightly but consistently below normal at every age, although histologic differences were slight. A microscopic study of the process of endochondral ossification in the tibia revealed decided differences between the



Proximal epiphyseal region of the tibia of male rats, H. and E. stain, × 75.
FIG. 1. Riboflavin control, 96 days of age. Spec. No. 8908, Plate 9426.
F.G. 2. Riboflavin control, 144 days of age. Spec. No. 8912, Plate 9427.
FIG. 3. Riboflavin-deficient rat, 96 days of age. Spec. No. 8896, Plate 9431.
FIG. 4. Riboflavin-deficient rat, 144 days of age. Spec. No. 8900, Plate 9429.

riboflavin-deficient and the control group.

Riboflavin Control Group. At 96 and 110 days the histologic aspect of the proximal epiphysis shows the normally thick surface cartilage, dense lamellar coat of bone, numerous coarse trabeculae, and a continuous layer of bone resting against the cartilaginous plate. The epiphyseal cartilage and subjacent diaphyseal area of the riboflavin control animal at 96 days of age are illustrated in Fig. 1. The trabeculae are numerous, close and coarse but do not extend far into the diaphysis. Few anastomoses are seen and erosion is very active. At 144 days (Fig. 2) the epiphyseal cartilage of the control animal shows a reduction in width, an increase of matrix and a more pronounced conical pattern of chondrocytes than usual for this age. The bone trabeculae are shorter and coarser and in many areas the marrow tissue is found in direct contact with the zone of enlarged cells. Many osteoclasts are seen on the surface of the bone trabeculae. In the more advanced age groups at 194, 237, and 278 days of age, the cartilaginous plate became progressively narrower but richer in matrix and the columnar arrangement of cells appeared still more irregular and frequently lost even the Dawsonian pyramidal aspect of normal old age. The bone trabeculae disappeared for the most part except for a few lying horizontally some distance below the disc. As contrasted with animals reared on a diet of natural foods[7] ossification was subnormal and trabeculation somewhat disturbed.

Riboflavin Deficient Group. At 96 days the epiphyseal surface cartilage was well-formed

[7] Beeks, H., Simpson, M. E., and Evans, H. M., *Anat. Rec.*, 1945, **92**, 109.

but the adjacent lamina of bone was so delicate as to be almost non-existent. The trabeculae were sparse in number and fine in structure. In the epiphysis, there was a thin, nearly continuous, horizontal layer of lamellar bone resting directly upon the cartilage (Fig. 3). The cartilaginous epiphyseal disc consists of 5 to 9 flattened cells and only one to 2 vesicular cells. The matrix is abundant in the basophilic region with bands occasionally traversing the entire width of the plate. Calcification of the matrix from the diaphyseal side is in progress and is enclosing the majority of vesicular cells. Osteogenesis has practically ceased and trabeculation consists only of short projections covered with true bone. This stage foreshadows the sealing-off of the epiphyseal cartilage from the marrow cavity by bone. Erosion is rare. The marrow shows an unusual wealth of osteoclasts but is normal in fat content.

At 110 days the epiphysis was somewhat larger; otherwise the appearance was the same as that of the 96-day-old tibia. The cartilage disc was slightly reduced in width. There was no evidence of fat infiltration in the marrow.

At 144 days the changes were more severe. The epiphysis showed advanced ossification. The bone lamina beneath the articulating cartilage, the trabeculae, and the layer of bone above the epiphyseal disc were increased in thickness. The cartilaginous disc (Fig. 4) is very narrow and the chondrocytes in the basophilic zone are arranged conically. The vesicular cells are enclosed in calcified matrix having a well-advanced marginal deposit of lamellar bone sealing it from the marrow. Three or 4 heavy isolated trabeculae extend into the diaphysis. Numerous osteoclasts are present in the marrow cavity and the number of large fat cells is increased markedly.

The findings in the tibias of the older age groups were similar. The margins of the epiphyseal cartilage were highly irregular and the matrix between the pyramidal groups of cells was increased. At 277 days the cartilage disc was the narrowest observed in this experiment and was reduced in a few places to 40 micra. The matrix lay in wide tracts in vertical or oblique planes across the disc, often penetrating the marrow on the diaphyseal side and forming short trabeculae which showed lamellar bone on their surface. Much of the disc was separated from the marrow by true bone. The 3 or 4 remaining lamellar trabeculae evinced no particular orientation with respect to the diaphysis. The marrow contained an unreduced amount of fat cells.

Discussion. The foregoing experiment was planned to study the effects of prenatal riboflavin deficiency on skeletal growth in the young. In contrast to Warkany, no skeletal abnormalities were observed at birth and the animals were sacrificed at ages varying from 3 to 9 months. The pathological changes observed were not necessarily progressive with age since the animals had varying degrees of the deficiency at the time of autopsy. Some of the older animals had lived considerably longer before showing outward signs of the deficiency and were less affected than animals autopsied at an earlier age. Also histological changes in the tibia due to age may have masked some of the changes due to riboflavin deficiency in the older animals. However, even at 278 days, the tibia of the deficient animal, although showing the characteristic changes of advanced age, differed markedly from that of the riboflavin control.

The principal histologic changes observed in the riboflavin-deficient group were retarded development of the epiphysis, progressive decrease in the width of the epiphyseal cartilage, increased hyalinization of its matrix, calcification and separation of the epiphyseal cartilage from the marrow cavity by a thin layer of bone. Osteogenesis ceased with an almost complete disappearance of the diaphyseal trabeculae. Unusual fat infiltration with reduction of hematopoietic tissue occurred in most of the older animals and was similar to the myeloid hypoplasia recently reported by Endicott *et al.*‡,[8] for acute riboflavin de-

‡ Endicott *et alia*[8] also found that both riboflavin and "folic acid" were concerned in this hypoplasia. Further studies will be necessary to separate the effects of these two vitamins on endochondral ossification.

[8] Endicott, K. M., Kornberg, A., and Ott, M., *Blood*, 1947, **2**, 164.

ficiency in the rat. In general, the changes observed in this study of the rat were similar but much more severe than those reported for the mouse by Levy and Silberberg.[2]

Reasons for the failure to duplicate the Warkany findings (*i.e.* multiple skeletal abnormalities) are not obvious. The experimental conditions and the purified high-carbohydrate diet were similar but not identical with those used by Warkany. The principal dietary difference was the use of an alcohol-extracted casein, probably containing slightly more riboflavin[9] than that used by Warkany, at a 24% instead of 18% level in the diet. The use of a high-fat diet to accentuate the deficiency should have overcome this difference in the protein components of the diets. At the present time 40 litters comprising 350 riboflavin-deficient young have been carefully examined at birth. While clearing with microscopic examination might have revealed some skeletal defects, abnormalities such as shortening of the mandible with protrusion of the tongue and syndactylism of the extremities which occurred with high frequency in Warkany's abnormal young could have been easily recognized by external inspection. Further studies on this problem are in progress.

Summary. The tibias of 6 male rats of the Long-Evans strain born from riboflavin deficient mothers and maintained on riboflavin deficient diets for 3 to 9 months were studied histologically and compared with an equal number of controls reared upon the same diet but with riboflavin added.

In the deficient rats the growth of the tibia was retarded and endochondral ossification was gravely impaired. Marked reduction in chondrogenesis and in capillary erosion and ossification were characteristic for all degrees of the deficiency. The formation of a thin calcified plate on the diaphyseal side of the epiphyseal cartilage is a finding in consonance with the early arrest of growth. Hematopoietic tissue was replaced by fat in all animals after 144 days on the deficient diet.

9 Cannon, M. D., Boutwell, R. K., and Elvehjem, C. A., *Science*, 1945, **102**, 529.

16179

A New Method for the Production of Non-Specific Capsular Swelling of the Pneumococcus.

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Neufeld,[1] and Neufeld and Etinger-Tulczynska[2] first described the phenomenon of capsular swelling of the pneumococcus when specific antiserum was added to the organism. This capsular swelling, known as the "quellung" reaction, is dependent upon the reaction of capsular polysaccharide with its specific antibody. It has been shown that an excess of specific polysaccharide,[3] heat,[4] papain digestion,[5] and repeated washing with water[6] can produce a reversal of the specific "quellung" reaction.

Non-specific capsular swelling of pneumo-

1 Neufeld, F., *Z. f. Hyg. u. Infectionskrankh.*, 1902, **40**, 54.

2 Neufeld, F., and Etinger-Tulczanska, R., *Ibid.*, 1931, **112**, 492.

3 Nungester, W. J., and Kempf, A. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 705.

4 Etinger-Tulczynska, R., *Z. f. Hyg. u. Infectionskrankh.*, 1933, **114**, 769.

5 Kalmanson, G. M., and Bronfenbrenner, J., *Science*, 1942, **96**, 21.

6 Kempf, A. H., and Nungester, W. J., *J. Inf. Dis.*, 1942, **71**, 50.

TABLE I.
The Effect of Various Proteins and Non-Protein Substances on Capsular Swelling of the Type 27 Pneumococcus.

Substance tested	pH 4.0	pH 5.0	pH 5.5	pH 6.0
Crystalline urease	4+*	0	0	0
Diaphorase	2+	0	0	0
3-Phosphoglyceraldehyde dehydrogenase	0	3+	4+	2+
Purified horse serum albumin	3+	0	0	0
" " hemoglobin	4+	0	0	0
Crude thrombin	4+	0	0	0
Zinc insulin	2+	0	0	0
Cytochrome C	0	0	0	0
" " (dialyzed)	0	0	0	0
Egg albumin	4+	0	0	0
Boiled horse serum albumin	3+	0	0	0
" " hemoglobin	4+	0	0	0
Commercial gelatin	1+	0	0	0
Wheat gluten	2+	0	0	0
Inositol	0	0	0	0
Inulin	0	0	0	0
Lactic acid, M/100	0	0	0	0

* Intensity of capsular swelling is indicated by the conventional notation from 0 to 4+ (maximum).

cocci was first reported by Lofstrom,[7] who used human sera obtained from patients in the acute phase of a febrile, usually bacterial, illness. When such sera were mixed with several different pneumococcal types, capsular swelling occurred. Type 27 pneumococcus was found to react most effectively. Similar acute-phase serum had been shown to produce a precipitate with "C" carbohydrate prepared from a rough strain of pneumococcus.[8-13] Both capsular swelling and the precipitin reaction are reversed by the addition of sodium citrate to remove ionized calcium.[12-14] Reciprocal-absorption experiments revealed that the same reactive protein produced a precipitate with "C" carbohydrate as produced pneumococcal capsular swelling.[13]

Recent work in this laboratory on the "non-specific capsular swelling" properties of human sera for Type 27 pneumococcus has revealed another mechanism for the production of a "quellung" reaction. As far as the author can discover, this mechanism has not been previously described. In the course of experiments to determine the effect of pH on the "non-specific capsular swelling" phenomenon of Lofstrom, acute-phase serum was treated with 0.01 M lactic acid. At pH 5.0-5.5 a protein fraction was precipitated that was partially soluble in an 0.01 M lactic acid solution adjusted to pH 4.0. When formalin-killed Type 27 pneumococcus was added to this soluble fraction at pH 4.0, capsular swelling was observed. All of the human sera tested, normal or abnormal, displayed this reaction. Studies on beef, swine, and lamb serum revealed a similar reaction.

Purified preparations of several enzymes, horse-serum albumin, and horse hemoglobin* were found to react effectively to produce capsular swelling at pH 4.0 (Table I). Test of serial dilutions of the albumin and hemoglobin solutions revealed that the minimal amount of the proteins required to produce "quellung" of the pneumococci was 120 and

7 Lofstrom, G., *Acta Med. Scand.*, 1943, Suppl. **141**, 1.

8 Tillet, W. S., and Francis, T., Jr., *J. Exp. Med.*, 1930, **52**, 561.

9 Tillet, W. S., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1930, **52**, 895.

10 Ash, R., *J. Inf. Dis.*, 1933, **53**, 89.

11 Abernethy, T. J., and Avery, O. T., *J. Exp. Med.*, 1941, **73**, 173.

12 MacLeod, C. M., and Avery, O. T., *J. Exp. Med.*, 1941, **73**, 183.

13 Lofstrom, G., *Br. J. Exp. Path.*, 1944, **25**, 21.

14 Carlens, E., *Acta Otolaryng.*, 1941, **29**, 316.

* Enzymes and purified albumin and hemoglobin were obtained through the kindness of Dr. Alexander Dounce, Department of Biochemistry, University of Rochester School of Medicine and Dentistry.

20 μg per ml, respectively, when enough organisms were added to give one or 2 pneumococci per oil-immersion field. Non-protein substances and cytochrome C (Table I) were non-reactive. In distinction to the other materials tested, 3-phospho-glyceraldehyde-dehydrogenase was optimally reactive at pH 5.5.

Inhibition of the "quellung" reaction could be brought about by the addition of readily ionized salts to the protein-pneumococcus mixture adjusted to pH 4.0. As low a concentration as 0.02 M sodium chloride and 0.07 M potassium iodide were effective in inhibiting capsular swelling. On the other hand, the reaction was not inhibited in 0.55 M glucose.

When purified horse hemoglobin was used to produce capsular swelling, it was noted that condensation of the protein occurred around the enlarged capsule. This gave the appearance of a distinct rim of golden pigment dotting the periphery of the capsule. As the reaction proceeded, each pneumococcus was completely surrounded by hemoglobin. Eosin azo-dog serum, in a similar manner, produced capsular swelling. The protein appeared to penetrate the capsule to give it a diffusely stained, deep-pink color. When specific antiserum was added to maintain capsular swelling and the pH was brought back to 7.0, the pink-staining protein was no longer present in the capsule. Pneumococci were treated with eosin azo-protein at pH 4.0, centrifuged free of the solution, brought to pH 7.0 and then placed in a colorless protein solution at pH 4.0. These organisms then contained no microscopically visible eosin azo-protein in the swollen capsular substance. The reaction would appear to demonstrate a reversible combination of protein with the capsular polysaccharide, dependent on conditions of pH.

This new method of producing non-specific capsular swelling is not peculiar to Type 27 pneumococcus. Formalin-treated pneumococci, Types 1, 2, 3, 5, 7, 8, 19, and 28 were tested and all reacted with equal facility to several proteins. Similar capsular swelling did not occur, however, with *Klebsiella pneumoniae*, a mucoid *Escherichia coli*, or a mucoid *Streptococcus hemolyticus*, although acid agglutination was observed under these conditions.

An attempt was made to determine the reactivity of purified polysaccharide substances with different non-specific proteins at pH 4.0. Both Type 28 and Type 3 polysaccharide substances were found to be reactive with proteins at pH 4.0 to produce a dense flocculent precipitate. This combination was reversible in that the precipitate washed in 0.01 M lactic acid solution adjusted to pH 4.0 and then brought to pH 7.0 was found to be clear and to react by precipitation with Type 3 specific antiserum. A pH of 4.0 was well below the iso-electric range of the proteins tested, and the pH of each protein-carbohydrate reaction mixture was redetermined to insure that flocculation was not due to iso-electric precipitation of the protein. The addition of sodium chloride did not inhibit precipitation, in distinction to the observed effect on the "quellung" reaction.

Discussion. The mechanism of the "quellung" reaction is not clear. Johnson and Dennison[15] felt that the increase in volume of the pneumococcus resulting from "quellung" was greater than the aggregate volume of the antibody molecules adherent to the capsule. They suggested that capsular hydration took place as a result of antigen-antibody combination. Mudd, *et al.*,[16] on the basis of electron microscopy, felt that the pneumococcal capsule consists of a gel of low density that reacts with homologous immune serum to produce increased thickness and density of capsular gel. Kempf and Nungester[6] noted that a 2 molar solution of sodium chloride did not prevent or reverse the "quellung" reaction to specific serum. The enlarged capsules disappeared on washing several times in water, only to reappear with the addition of physiological saline solution.

Summary. The experiments outlined indicate that pneumococcal polysaccharides combine with various proteins at pH 4.0 to form

15 Johnson, F. H., and Dennison, W. L., *J. Immunol.*, 1944, **48**, 317.

16 Mudd, S., Heinmets, F., and Anderson, T. F., *J. Exp. Med.*, 1943, **78**, 327.

insoluble aggregates. This reaction is one of loose combination, since it can be reversed by readjusting the pH to 7.0. Non-specific capsular swelling of pneumococci can be produced also under these conditions, but, in addition, the protein-pneumococcus mixture at pH 4.0 must be relatively salt-free. It is felt that an altered state of the capsular gel is produced by the interaction of protein and carbohydrate. In the absence of ionized salts, the polysaccharide-protein gel becomes increasingly hydrophilic and therefore capable of enlarging to produce the "quellung" reaction. Both specific and the described acid-protein quellung can be produced in the absence of calcium in distinction to Lofstrom's non-specific "quellung" phenomenon.

Grateful acknowledgment is made to Henry W. Scherp, Ph.D., Department of Bacteriology, University of Rochester School of Medicine and Dentistry, for supplying the polysaccharide preparations and eosin azo-protein, and for many helpful criticisms and suggestions.

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Effect of Alloxan Diabetes on Reproduction in the Rat.*

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The administration of alloxan to experimental animals will produce a condition analogous to diabetes mellitus in man.[1,2,3] The principal effect of the drug is a selective necrosis of the islets of Langerhans in the pancreas, although there is slight damage to other tissues.[4,5] The experimental diabetes may be transitory in character and the animal recover, or it may be permanent, becoming progressively more severe until the animal succumbs. The dose of the alloxan administered is the most important factor in the ultimate course of the diabetic state.

Friedgood and Miller[6] injected alloxan 4 days before parturition and obtained normal litters. This observation suggests that terminal diabetes is not sufficient to interfere with the development of fetuses. The high incidence of pregnancy complications in human diabetics prompted us to restudy the problem and the following experiments were designed to provide additional pertinent information.

Methods. The objectives of these experiments were three-fold: (1) to determine the effect of alloxan diabetes on cyclical activity and fertility in the rat, (2) to study reproduction in rats made diabetic early in pregnancy and (3) to evaluate the course of pregnancy and labor in the rat with diabetes controlled adequately by insulin.

Young mature female rats, weighing 175 to 250 g, of the inbred Sprague-Dawley stock raised and maintained in our laboratory were used throughout these studies. They were given free access to a balanced diet which was supplemented by milk as well as wheat germ oil once a week. Sex cycles were followed routinely employing the standard vaginal

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

1 Bailey, C. C., and Baily, O. T., *J. A. M. A.*, 1943, **122**, 1165.

2 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

3 Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

4 Brunschwig, A., and Allen, J. G., *Cancer Res.*, 1944, **4**, 45.

5 Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet*, 1943, **1**, 484.

6 Friedgood, C. E., and Miller, A. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 61.

smear technique. The animals were mated by introducing them to males of known fertility and the onset of gestation noted by the presence of spermatozoa in the vaginal smear. It was further checked by the appearance of the placental sign on the twelfth day of the gestation.

Fasting blood sugar levels on venous blood from the tail were determined at 2- or 3-day intervals throughout these experiments by Somogyi's micro-modification of the Schaffer and Hartmann method. The animals were placed in metabolism cages during the day at least 4 hours prior to the blood sugar determinations in order to insure fasting levels. In those animals receiving insulin for the control of the hyperglycemia, qualitative sugar determinations on the urine at frequent intervals made it possible to follow the course of the diabetes.

Cyclical activity was studied in the first group of 6 animals. After a baseline of normal estrous cycles was established for each animal, it was injected with alloxan intravenously. The vaginal smears were followed for a sufficiently long period so that the character of the diabetes induced in the animal could be established and the effect of the hyperglycemia on estrous cycles studied. Varying amounts of alloxan were used. Some of these animals were subsequently mated and their pregnancies followed.

A second group of 49 normal female rats was placed with males and as soon as pregnancy was diagnosed by the appearance of spermatozoa in the vaginal smear they were segregated. Prior to the fifth day of the pregnancy they were injected with varying amounts of alloxan. Blood sugar determinations were made on alternate days throughout their pregnancies. Three of these animals were followed through a second pregnancy. The animals were carefully observed for the accidents of pregnancy.

A third group of 7 rats was made diabetic by alloxan. When it was certain that the diabetes was permanent in character, the hyperglycemia was controlled by insulin. These animals were allowed to mate and the resultant pregnancies carefully studied.

Results. The Effect of Alloxan Diabetes on the Estrous Cycle. After the normal estrous cycle had been established in 6 rats, each received 40 mg of alloxan per kilo of body weight. One animal died 3 days after the injection at which time the fasting blood sugar value was 379 mg %. Two of the animals developed a transient diabetes only and in these the blood sugar levels returned to the normal by the eighth day. The hyperglycemia in these animals reached their highest values, 160 and 207 mg %, on the fifth day following the administration of alloxan. The remaining 3 animals developed permanent alloxan diabetes. Fasting blood sugar levels ranged from 288 to 360 mg % throughout the period of observation.

Following the development of hyperglycemia the estrous pattern changed. Estrous smears occurred with considerable irregularity and the interval was prolonged. The normal 4 or 5 day pattern was replaced by one of 9 to 12 days, the animal remaining in the diestrous condition the greater part of each cycle.

Effect of Alloxan on Mating. Nine diabetic animals in whom blood sugar levels vary from 225 to 350 mg % were placed with males of known fertility. When estrus occurred these animals mated normally as evidenced by the presence of spermatozoa in the vaginal smear. However, the irregularity of the estrous cycles and the prolonged interval between estrous periods lengthened the time necessary for mating.

Three of the animals died 4 or 5 days after spermatozoa were found in the vagina. These animals had normal gestations and their deaths were probably due to the diabetes. Two rats who had hyperglycemia prior to pregnancy developed normal blood sugar levels during the gestation. These 2 animals carried their pregnancies to term and delivered normal litters uneventfully. The remaining 4 animals continued to exhibit a marked hyperglycemia with levels ranging from 210 to 360 mg %. The placental sign appeared on the twelfth day of the gestation. However, at the end of their pregnancies they delivered macerated placentas and no fetuses.

TABLE I.
Summary of Diabetic Rats Treated with Protamine Zinc Insulin. Note the lack of difference in size of litters or weights of fetuses between normal and diabetic animals

Rat No.	Units of Protamine zinc insulin/day	No. of fetuses in litter	Sex		Avg wt, g		Blood sugar P.P. No insulin mg %	Remarks
			M	F	M	F		
372	0.75	9	4	5	6.2	4.5	215	F.B.S. 2 days postpartum
470	0.75	5	2	3	5.0	4.0	373	" " "
471	0.75	11	4	7	6.2	5.0	360	" " "
580	0.75	4	2	2	5.5	6.0	270	" " "
269	0.75	5	2	3	6.5	6.0	399	" " "
183	0	9	4	5	6.2	6.2	Normal	Breeding stock
112	0	6	4	2	5.2	5.0	"	" "
92	0	10	4	6	5.0	5.0	"	" "
100	0	5	2	3	6.7	5.6	"	" "
186	0	4	2	2	5.6	5.6	"	" "

Effect of Alloxan on Pregnancy. A total of 49 pregnant rats were injected intravenously with alloxan, the amount ranging from 30 to 60 mg per kilo, prior to the fifth day of gestation. The most satisfactory dose from the viewpoint of developing a permanent diabetes was 40 mg per kilo. Twenty-seven of these animals died at various stages of their pregnancies and they were studied postmortem. Fourteen of the animals were sacrificed at various periods in their pregnancies in order to provide fresh material for histologic study. Five of the animals survived their pregnancies and delivered macerated placentas at term. The remaining 2 animals did not maintain their hyperglycemia during the pregnancy and they were sacrificed on the ninth and fourteenth days of the gestation. Their pregnancies were progressing uneventfully.

Gross and histologic examinations of the reproductive organs of the diabetic rats revealed typical changes in the entire group. The pregnancies progressed normally until the twelfth day of the gestation. The fetuses and the placentas were normal. Following this period the fetuses died and were slowly absorbed. Necrotic fetuses in various stages of degeneration and absorption were present in the animals sacrificed at varying periods of pregnancy. (Fig. 1.) The placentas continued to grow after fetal death and remained attached to the uterine wall until separated during parturition. The size of the placenta at term was distinctly smaller than the normal placenta probably because of the absence of the fetus. The placentas were delivered at term after an uneventful parturition and all visible remnants of the fetus had disappeared.

These experiments provided proof that the rat made permanently diabetic by the administration of alloxan very early in pregnancy could not deliver a living litter at term because of fetal death prior to the twelfth day. The next step was to see if the control of the hyperglycemia by insulin would result in normal pregnancies. A group of 7 rats was made diabetic by the intravenous injection of 40 mg of alloxan per kilo of body weight. Their fasting blood sugar levels ranged from 253 to 458 mg %. Two weeks later when it was made certain that the diabetes was permanent the animals were placed on protamine zinc insulin, the amount administered being controlled by daily qualitative urine sugar determinations. When the diabetes was controlled by the insulin, the animals were allowed to mate and become pregnant.

The most striking result following the control of the diabetes by insulin was the resumption of normal, regular estrous cycles. The animals became pregnant, carried through to term uneventfully and delivered normal living litters. Two of these animals died at term and subsequent autopsy examinations did not reveal the cause for the deaths. The placentas and the young were normal.

Insulin therapy was discontinued several days after delivery in order to determine the status of the diabetic state. In all instances blood sugar values returned to their previous levels at which they continued as long as

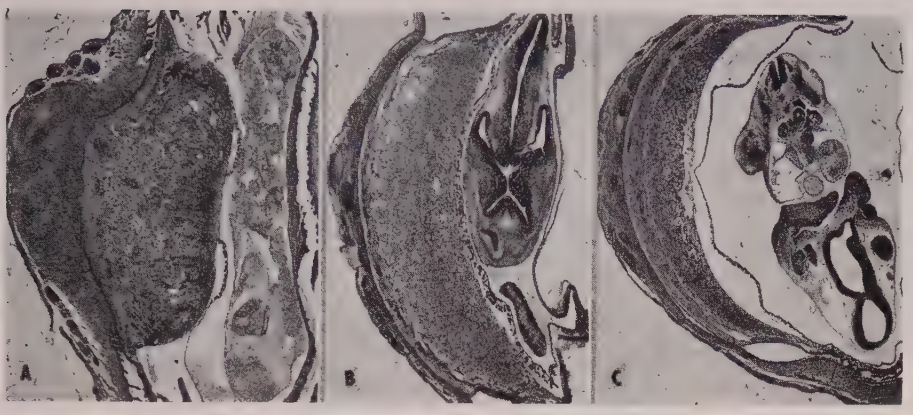

FIG. 1.

Photomicrographs through gravid uteri. A. Permanently diabetic rat at 16 days of gestation. Note the necrotic condition of the fetus. B. Rat with transient diabetes at 15 days gestation. The diabetes lasted 7 days. The fetal structures appear normal. C. Control animal at 14 days gestation.

no insulin was administered. The pregnancies and the insulin therapy did not alter the diabetes. (Table I).

Discussion. Alloxan diabetes in the rat alters the normal cycles, decreases fertility because of this change and affects pregnancy seriously. It is important to make certain that the permanent diabetic state has been induced. Transitory hyperglycemia is a frequent result following an inadequate amount of alloxan. Some of the animals used in these experiments recovered from their diabetic state early in pregnancy and subsequently delivered normal litters at term. Fasting blood sugar levels vary considerably and in the animals used in this study they ranged from 186 to 425 mg %.

Living litters were delivered in none of these diabetic animals. Characteristically, the pregnancy progressed normally until the midpoint of the gestation when the fetus succumbed, following which it was slowly absorbed so that it was absent at term. The placentas remained attached and were delivered at term following a fairly normal parturition.

These observations suggest that terminal diabetes is not sufficient to interfere with the development of the fetuses and that the critical period for the fetus was midpregnancy.

Is it the hyperglycemia that interferes with the estrous cycles and normal gestation in the rat? If the effect of insulin is simply the control of the hyperglycemia, this may be so. A group of diabetic rats were adequately controlled by insulin following which the estrous cycles returned to normal, they conceived and delivered normal living litters at term. The weight of the individual fetus and the gross weight of the litter compared favorably with normal control animals in the colony. It is possible that insulin does more than neutralize excessive blood sugar and diabetes represents more than an altered carbohydrate metabolism.

Summary and Conclusions. The effects of alloxan diabetes on ovarian activity and pregnancy were studied in a series of 63 adult female rats. The development of permanent hyperglycemia resulted in an alteration of the normal estrous pattern, so that the intervals were greatly prolonged. Pregnancy progressed normally until about the twelfth day following which the fetus died and was slowly absorbed. The placentas were retained and were delivered on the day of parturition. The adequate treatment of the alloxan diabetes by insulin resulted in normal pregnancies and live litters delivered at term.

16181

Effect of Dietary Variations upon the Toxicity of DDT to Rats or Mice.*

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The effectiveness of toxic agents in animals frequently depends upon the composition of the basal diet fed. Thus a high protein diet has been found to protect the liver against such diverse toxic agents as arsphenamine,[1] p - dimethylaminoazobenzene,[2] chloroform,[3] and selenized wheat.[4] Methionine is effective against several of these agents.[5] Another nutrient reported to modify toxicity is fat. When rats were fed trinitrotoluene (TNT) in diets high in protein or carbohydrates, little or no ill effects were observed, although definite toxic effects resulted when a high fat diet was fed.[6] On the other hand, mice fed dinitrotoluene (DNT) grew better and lived longer on a high fat diet than when the basal diet was nearly devoid of fat.[7] Vitamin C is reported to be of value in the prevention and treatment of toxic effects of TNT in munition workers[8] and to aid in the detoxification of aniline in rabbits and mice.[9] Chloronitrobenzene toxicity has been found to be augmented by fats and oils, as well as by alcohol,[10] and alcohol also increases the toxicity of dinitrotoluene.[7,10] These observations and the widespread use of DDT (2,2 bis(p-chlorophenyl) 1,1,1 trichloroethane) as an insecticide raised the question whether the toxicity of this compound in higher animals might not also vary with the composition of the basal diet fed.

Experimental. Methods. Most of the experiments were performed on young mice, but adult mice and both young and adult rats were also used in certain studies. The animals were kept in screened cages in groups of 3 to 6 and they were fed the synthetic or semisynthetic diets listed in Table I, or similar diets in which the percentage of fat or protein was altered isocalorically at the expense of the carbohydrate. The animals were weighed periodically and the toxicity symptoms noted. The DDT used in these experiments was prepared by the method of Darling[11] and recrystallized twice from ethyl alcohol.

Results. In preliminary studies, DDT was incorporated into the yeast diet I and fed to young mice in graded concentrations ranging from 0.01% to 0.3%. At concentrations of 0.05% and over, the mice rapidly developed toxicity symptoms and died within 2 weeks or less. At the lowest concentration, 0.01% they survived for a 9-week period. At the intermediate concentrations, 0.03% and 0.04%, approximately one-half of the animals lived for 4 weeks, depending upon the concentration of the insecticide and the size of the mice. Older mice resisted the effects of the toxic agent somewhat better than younger ones. The symptoms of toxicity were similar

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and the Jonathan Bowman Cancer Fund.

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TABLE I.
Composition of the Diets Fed.

Yeast diet (I)	g	Synthetic diet (II)	g
Crude casein	18	Crude casein	20
Wesson's salts[14]	4	Wesson's salts	4
Brewers yeast	8	Celluflour	2
Corn oil	5	Corn oil	5
Dextrin	70	Dextrin	69
			mg per 100 g of diet
		Pyridoxine hydrochloride	0.5
		Thiamine chloride	0.6
		Calcium pantothenate	2.0
		Riboflavin	0.6
		Nicotinic acid	1.0
		Choline chloride	100.0
		Inositol	50.0
		p-Aminobenzoic acid	30.0
		Cystine	100.0

to those described by others[12,13] and were characterized by hyperexcitability, followed by a jerking or retraction of the head, which developed into a general tremor that increased in intensity until there was complete loss of control, convulsions, and death. Alopecia was common in certain series. The compound did not exert any appreciable effect on the rate of growth until the tremors became sufficiently severe to interfere with the ability of the mice to eat. In general, rats responded to DDT like mice, although higher concentrations of DDT were necessary before deaths resulted in a comparable period of time: 0.15% of DDT was about as effective in a rat as 0.04% in the mouse.

The effect of DDT against either species depended upon the percentage of fat in the basal diet (Table II). Mice invariably developed toxic symptoms and died sooner when fed a diet containing 5% or more of fat than when fed a diet containing only 0.5% of fat; this was observed in both young and older mice and in series in which the concentration of DDT was 0.03% or 0.04% (Table II). Similar results were obtained with rats fed 0.15% of DDT (Table II). In other experiments, series of different fats were fed to mice at a 15% level in diets containing 0.03% or 0.04% of DDT. All fats tested appeared to increase the sensitivity of mice to DDT at this level. Mice fed a completely saturated fat, hydrogenated coconut oil, developed tremors and died at about the same time as those ingesting the moderately saturated fats, butter or lard, or the highly unsaturated fats, peanut oil or corn oil. The effect of the fat was not modified by the addition of 0.5% of cholesterol.

A reduction in the level of protein in the diet to 10% apparently decreased the resistance of the mice to DDT. This effect was noted whether the percentage of fat in the diet was high or low (Table II). When the level of protein in the diet was increased above 20%, the results were variable although some protective action against DDT appeared in certain series.

Discussion. The greater sensitivity of animals to DDT fed in a high fat diet as compared to one low in fat seems to be primarily due to a greater efficiency of absorption of DDT on the high fat diets. DDT is readily soluble in fat and tends to concentrate in the fat depots of animals exposed to this insecticide, *e.g.*, in rats,[15] cows,[16] and dogs.[17]

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TABLE II.
Effect of the Level of Fat and Protein in the Diet upon the Survival of Mice and Rats Fed DDT.
Diet II.

Casein, %	Corn oil, %	Number of animals alive at: 0 wk	1 wk	2 wks	3 wks	4 wks	5 wks	6 wks
		0.03% DDT, young mice.						
20	0.5	4	4	4	4	4	4	4
20	5	4	4	4	4	4	3	2
20	15	4	4	3	2	2	1	0
		0.03% DDT, young mice.						
20	0.5	5	5	5	5	3	3	
20	15	5	5	5	4	2	1	
10	15	5	5	4	1	1	0	
50	15	5	5	4	4	4	4	
		0.04% DDT, young mice.						
20	0.5	10	10	7	6			
20	5	4	4	1	1			
20	15	10	7	3	0			
10	0.5	6	5	1	0			
10	15	6	6	1	0			
		0.04% DDT, adult mice.						
20	0.5	4	4	4	4	4	4	4
20	5	4	3	3	3	3	2	2
20	15	4	4	4	4	4	3	2
20	25	4	3	3	2	1	1	1
10	15	4	3	0	—	—	—	—
50	15	4	3	3	3	3	2	2
		0.15% DDT, young rats.						
20	0.5	6	6	5	5	5		
20	5	6	2	0	—	—		
20	15	6	4	1	0	—		

More DDT accumulated in the fat of dogs when the compound was fed in corn oil solution than as a solid[17] and the symptoms of toxicity due to injected DDT were also more severe,[12,13] when an oil solution was used. Factors affecting the translocation of DDT within the body are probably more important in determining resistance to DDT than to other poisons. DDT does not appear to be rapidly detoxified in the body[18] and its association with fat may be an important mechanism for its removal from the general circulation. However, at least one metabolic derivative of DDT (di(p-chlorophenyl) acetic acid) has been identified in the urine of rabbits,[19] and fat may affect the speed with which such metabolites are formed. Dietary fat seems to influence the toxicity of certain nitro compounds.[6,7]

The mouse appears to be more sensitive to DDT than the rat, for on comparable diets tremors produced in mice fed 0.03% to 0.04% of DDT were similar to those in rats fed 0.10% to 0.15% of DDT. In other words the rat can tolerate 3 times the concentration tolerated by the mouse. However, the mouse consumes roughly 2 to 3 times as much food per unit of body weight as the rat and, hence, the amounts of ingested DDT per gram of body substance required for the production of tremors were quite similar in the two species. Indeed, it has been reported that the rat is somewhat more susceptible than the mouse to injected DDT or to large single oral doses.[12] Nevertheless, the present data indicate that the mouse has definite advantages as an animal for the bio-assay of foodstuffs exposed to the insecticide.

Summary. Mice fed 0.03% to 0.04% of DDT in a medium fat diet (5%) developed toxic symptoms and died; 0.10% to 0.15% of DDT in the same diet was necessary to

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[19] White, W. C., and Sweeney, T. R., *Public Health Rep.*, 1945, **60**, 66.

produce a similar toxicity in the rat.

A reduction in the level of dietary fat to 0.5% decreased the toxicity of DDT in both species; when the diet contained 15% of fat, the symptoms of toxicity were aggravated. Several different types of fat were essentially equal in aggravating the toxicity.

On a low protein diet (10%) the toxicity of DDT was increased somewhat; whereas on a high protein diet (30%) the effects were variable.

16182 P

Endocrine Interrelationship and Spontaneous Tumors of the Adrenal Cortex in NH Mice.*

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Adenomas of the adrenal cortex appear in several inbred stocks of mice following gonadectomy.[1,2,3] In certain stocks, however, the phenomenon does not occur following removal of the primary sex glands.[3,4] In only one strain have similar adenomas been observed to appear spontaneously in non-castrate animals.[5] This is the NH stock† (Minnesota subline); tumors occur primarily in females. In these mice adenomas of the same type which develop spontaneously can be induced to appear precociously by gonadectomy.[6] The purposes of the current studies on this stock are to determine first, whether "physiologic castration" is responsible for the induction of spontaneous adenomas of NH females (spontaneous cortical tumors are very rare in males) and, second, to investigate the effect of gonadotrophic hormone on the secretion and development of these tumors.

The vaginal smear which is exhibited by mice with cortical adenomas is typical. The vaginal secretion is copious, relatively watery, and has a high cellular content. The cells are of two general classes—epithelial cells and leukocytes—these are approximately equally distributed. The epithelial cells may be classified as cornified and non-cornified. The ratio of cornified to non-cornified cells in the untreated tumor-bearing animal is 25 to 75.

The estrous cycle was studied in 6 inbred stocks of mice (CBA, dba, Balb, Strong A, F., NH). In general, it was found that the estrous cycle lengthened as the animals became infertile. This was observed to occur earlier in the NH stock than in any of the others except the F mice. Females of all stocks except the NH and F were cycling beyond one year of age. The F females (10 mice) exhibited a castrate smear by one year of age but did not develop cortical adenomas. If castrated early in life these mice showed only a minimum tendency towards adenoma development, suggesting that the end organ is not sensitive to the stimulus towards tumor formation. (CBA, dba, and Balb mice exhibit cortical adenomas within 3-5 months after gonadectomy). In most cases the NH females (43 mice) did not

* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the National Cancer Institute, and the Cancer Fund of the Graduate School of the University of Minnesota.

† This stock was obtained in the 8th inbred generation from Dr. L. C. Strong of the Yale University School of Medicine.

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develop castrate smears, but developed the smear characteristic of mice bearing cortical tumors within a few cycles after lengthening of the cycle (within 30-50 days after the last litter the vaginal smear picture typical of cortical adenoma appeared).

Histologic study of the ovary revealed that at one year of age Graafian follicles were present in the ovaries of all stocks except the NH. (The reason for lack of cycles in the F strain is not clear). The evidence suggests that physiologic castration may be a factor in the development of adenomas in the NH females. It is of interest that the smear typical of tumor formation appeared before there was histologic evidence for a well developed tumor. Sections of the vagina showed extreme cornification. It is probable that the histologically unaltered NH adrenal may secrete estrogen in quantity sufficient to stimulate the female reproductive tract.

When only the ovaries were removed from 6 tumor-bearing mice, the vaginal smear picture was unaltered. When the adrenals alone were removed‡ (ovaries remaining) then the vaginal smear became of castrate type within 5 days (7 cases). This constitutes proof that the adrenal tumors were the primary source of estrogen.

‡ Animals maintained on aqueous cortical extract—Upjohn.

The effect of gonadotrophic hormone was studied on the same mice (adrenalectomized or ovariectomized). One hundred and ten international units (5 units daily for 22 successive days) of pregnant mare serum§ elicited no change in the castrate smear of the adrenalectomized NH females, indicating the refractoriness of the ovaries to gonadotrophic hormone (the ovaries of older mice of other stocks were readily stimulated). The same amount of this hormone, when given to 3 ovariectomized mice (with adrenal tumors) induced a change in the vaginal smear picture: (1) the quantity of secretion was increased, and (2) the ratio of cornified to non-cornified cells was altered from 25:75 to at least 50:50. The number of leukocytes remained the same. It would appear that gonadotrophic hormone can influence sex-hormone secretion of an adenoma of the adrenal cortex.

Summary. The occurrence of spontaneous estrogen-secreting tumors of the adrenal cortex can be correlated with early cessation of ovarian activity in the NH stock. Estrogen is probably secreted by the adrenal cortex even preceding adenoma formation. Gonadotrophic hormone enhanced estrogenic secretory activity of cortical adenomas.

§ Gonadogen—Upjohn.

16183

Early Effect of X-rays on Ovaries of Normal and Adrenalectomized Rats.*

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In a previous paper[1] we described the changes occurring in the rat ovary during the first few hours after irradiation with X-rays.

* Aided by a grant from the British Empire Cancer Campaign.

1 Halberstaedter, L., and Ickowicz, M., *Radiology*, 1947, **48**, 369.

These alterations are characterized by the presence of numerous pycnotic nuclei of the follicular cells of the granular layer of the follicle.

We are able to distinguish between pycnotic nuclei induced by irradiation and those normally found in certain ovarian follicles, by showing that pycnotic nuclei of granular cells

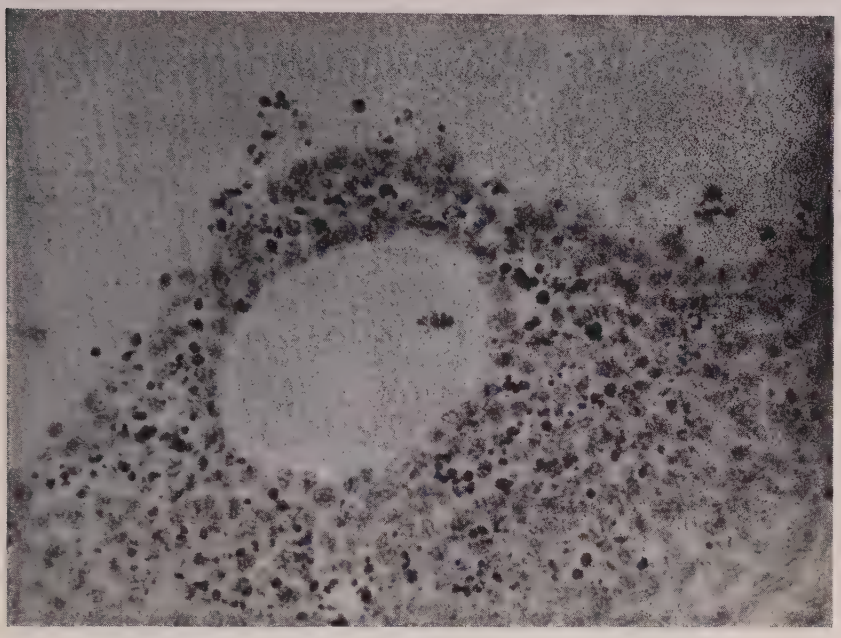

Fig. 1.
Adrenalectomized rat. Graafian follicle with numerous pycnotic nuclei. The nucleus of the ovum is in mitosis. × 640.

in the ovaries of non-irradiated rats occur only in follicles in an advanced stage of development, and whose ovules are already dividing, whereas after X-irradiation numerous pycnotic nuclei are also found in very young follicles and in those whose ovules are in the resting state. In studying the earliest ovarian lesions induced by irradiation it is therefore necessary to examine the entire organ microscopically by means of complete serial sectioning, in order to determine the condition of the ovule.

In addition, we have observed that during the first few hours after irradiation ovarian lesions are limited particularly to follicular cells, while no lesions are to be seen in the other elements of the ovary.

The object of the present study was to determine the effect of adrenalectomy on pycnosis of the follicular cells of the rat ovary during the first few hours after X-ray irradiation.

Methods and Technique. Our experiments on the ovaries of highly inbred albino rats (weighing approximately 150 g) fall into the following 3 categories:

A. Adrenalectomized rats. These animals were killed 24 hours after double adrenalectomy.

B. Irradiated rats. These animals were irradiated over the abdominal region with 2000 r and were killed 4 hours after irradiation.

C. Adrenalectomized and irradiated rats. These animals were adrenalectomized and were irradiated over the abdominal region with 2000 r 24 hours later. They were killed 4 hours after irradiation.

Irradiation was delivered by a Machlett X-ray tube operated on a multivolt apparatus of 150 K.V., Al. 0.5 mm, distance 30 cm, intensity 100 r/minute. The ovaries were fixed in Bouin's fluid. Complete serial sections were prepared and stained with iron hematoxylin and eosin, according to the method of Masson.

Histological Description. The following microscopic description is based on the composite data for each group of rats, with special emphasis on pycnotic nuclei of the follicular cells and their relationship to the state of the ovule.

A. *Adrenalectomized rats.* Follicles at differ-

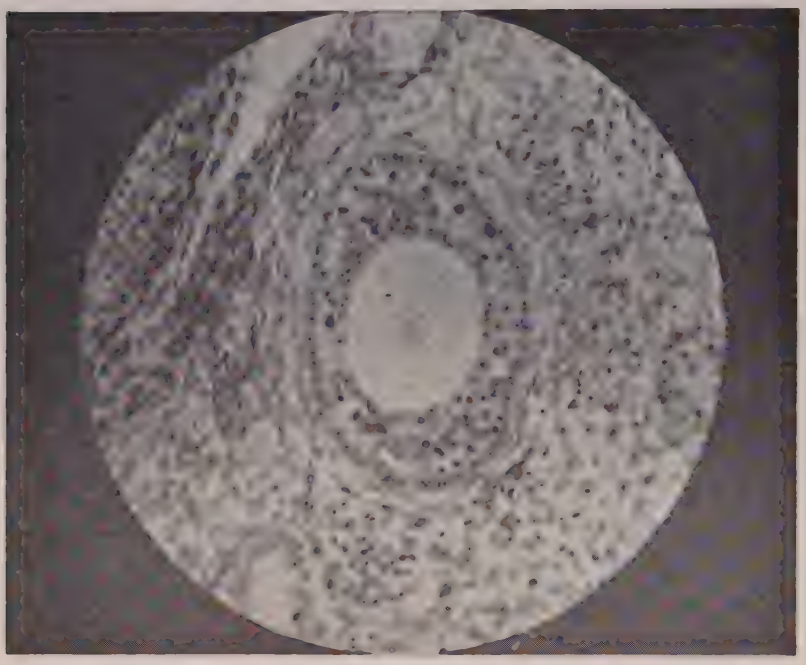

Fig. 2.
Rat irradiated with 2,000 r. Young follicle with pycnotic nuclei. × 450.

Fig. 3.
Rat adrenalectomized and irradiated with 2,000 r. Young follicle without pycnotic nuclei. × 450.

ent developmental stages were found: Graafian follicles, as well as stratified and young ones. The Graafian follicles of varying stages may be divided into 2 groups: a) Those with pycnotic nuclei in the granular layer, and with a dividing ovule (Fig. 1); and b) those without pycnotic nuclei or with very few in the granular layer and with a resting ovule.

We have, however, come across one ovary with Graafian follicles containing resting ovules, but with a considerable number of pycnotic nuclei in the granular layer. Such follicles are very unusual. The presence of pycnotic nuclei in the granular layer of young follicles (from primary to stratified follicles) is likewise exceptional. On the contrary, mitoses are frequently seen in the granular layer of young follicles.

Furthermore, no changes associated with adrenalectomy have been observed in the other ovarian elements, such as the atresic follicles, corpora lutea, blood vessels or the general stroma. Thus, the ovaries of adrenalectomized rats 24 hours after the operation do not differ significantly from normal rat ovaries.

B. *Irradiated rats.* Microscopic examination 4 hours after X-ray irradiation revealed extensive pycnotic lesions of the follicular cells in all advanced as well as in young follicles (Fig. 2). The number of atresic follicles seems to have increased. However, very rarely, some Graafian follicles appear to be resistant to X-rays; but these are exceptional cases and do not alter the general picture of the massive pycnotic lesion observed in the ovaries 4 hours after irradiation with an adequate dose.

C. *Adrenalectomized and irradiated rats.* Microscopically no pycnotic nuclei were seen in cells of the granular layer in young follicles (Fig. 3) and in the majority of Graafian follicles containing resting nuclei. As in the ovaries of normal rats, numerous pycnotic nuclei were seen in follicles with dividing ovules. However, we have also observed pycnotic nuclei in certain follicles containing resting ovules in the ovaries of one rat. But these lesions were, nevertheless, infinitely less pronounced than those observed in irradiated rats without previous adrenalectomy. No changes were seen in the other ovarian elements.

Discussion. Our experiments demonstrate that adrenalectomy inhibits the appearance of pycnotic lesions in the rat ovary 4 hours after X-ray irradiation. These observations are analogous to those obtained with lymphatic tissues and described by Leblond and Segal[2] and by Halberstaedter and Ickowicz.[3]

The small cells in the granular layer of the ovary appear to react as do lymphocytes. We are as yet unable to tell just how far this analogy may be drawn, since our experiments deal only with pycnotic lesions due to X-ray irradiation. Actually, observations on lymphocytes have shown that these cells are extremely sensitive not only to X-rays but also to a number of harmful agents, both chemical and physical[4] and even to so simple a trauma of the animal organism as an incision of the skin.[5] In any case our experiments clearly demonstrate that under certain conditions, such as X-ray irradiation for example, the adrenal glands influence the pycnotic reaction of the cells of the granular layer of the ovary.

An important point brought to light by our experiments is the fact that adrenalectomy inhibits pycnosis of the granular cells of the ovary even after direct irradiation of the organ. This constitutes a special phenomenon, since in the case of lymphocytes adrenalectomy seems to influence the ensuing pycnosis caused only by the indirect effect of the irradiation.[2,3] So far we have not yet elucidated the question of the influence of adrenalectomy on the indirect effect of irradiation on the ovary.

Summary. The pycnotic lesions of the cells in the granular layer of the ovarian follicles of rats 4 hours after X-ray irradiation are inhibited by previous adrenalectomy.

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2 Leblond, C. P., and Segal, G., *J. Roentgenol. and Rad.*, 1942, **47**, 302.

3 Halberstaedter, L., and Ickowicz, M., *Radiologia Clinica*, 1947, **16**, 240.

4 Selye, H., *J. Clin. Endocrin.*, 1946, **6**, 117.

5 Ickowicz, M., *Radiologia Clinica*, 1947, **16**, 231.

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Aluminum Penicillin in Mouse Protection Tests.

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In searching for a preparation of slowly absorbed penicillin that would give satisfactory results, the amorphous aluminum salt of penicillin was prepared in our laboratories. As expected, this penicillin was found to be highly insoluble in water. It was therefore thought that if it could be suspended in a suitable non-aqueous medium a single daily parenteral injection would give prolonged therapeutic activity. Peanut oil was selected as the vehicle for suspension.

It is the purpose of this report to show the protective effect of aluminum penicillin in peanut oil on mice infected with *Diplococcus pneumoniae*. Clinical reports to be published elsewhere will describe blood level determinations and therapeutic efficacy of aluminum penicillin in human subjects. However, when 300,000 units of aluminum penicillin in peanut oil is given intramuscularly to human subjects, blood levels of 0.03 units per ml of serum were found to persist from 12 to 24 hours. The concentration of penicillin in body fluid of treated mice has not been determined.

Procedure. 18 to 22 g mice were infected intraperitoneally with 0.5 ml of a 10^{-5} dilution of an 18-hour broth culture of *Diplococcus pneumoniae* Type I (SVi strain) containing 1000 MLD. Two hours later treatment was begun as indicated in Table I by injecting 0.1 ml of the suspension of penicillin in peanut oil intramuscularly. The suspensions were made by diluting penicillin in oil and mixing in a Waring blendor to the proper unitage. All dilutions were assayed before use. Treatment was repeated daily for 4 days and mice were held until the eighth day when the results were finally determined.

Under the conditions of these experiments, the effectiveness of aluminum penicillin was compared with calcium penicillin in peanut oil and sodium crystalline penicillin in peanut oil and beeswax (4.8 W/V). Untreated, infected controls died within 36 hours. The results are shown in Table I.

From the above data it is determined that the PD_{50} for each of the compounds tested is:

(1) Aluminum penicillin in peanut oil	34 units
(2) Sodium penicillin in oil and wax	40 units
(3) Calcium penicillin in oil	95 units

This suggests that under the conditions described, aluminum penicillin is superior in protective ability to either of the other compounds tested. This may be considered surprising in view of the reference to human blood levels in which aluminum penicillin does not appear to remain in the blood stream as long as peanut oil and beeswax preparations. Further studies are in progress to explain this seeming inconsistency, however, it seems appropriate at this time to propose an explanation. Aluminum penicillin being very insoluble in water must, for purposes of assay or utilization by the animal body, be converted into a soluble salt, *e.g.* sodium penicillin. This process takes place *in vivo* slowly and at a rate proportional to the amount of aluminum penicillin present in tissues. For this reason blood serum levels run a curve having a peak about one hour after injection, slowly diminishing in several hours to a level below an amount that can be measured by present methods for determining its concentration. This lower concentration, though at present not considered therapeutic, may be adequate to hold the invading organisms in check until the body defenses can overcome the infection.

To further study the prolonged action of aluminum penicillin in oil against pneumococcal infections in mice, infected animals were treated with a single intramuscular injection. Results of these protection tests are given in Table II.

Conclusion. Aluminum penicillin in oil ef-

TABLE I.
Protection with Multiple Doses of Penicillin.

Preparation	Units of penicillin: Daily	Units of penicillin: Total 4 days	Survivors: No. of mice	Survivors: 8 days	Survivors: % protection
Aluminum penicillin in oil	200	800	80	80	100
	150	600	50	50	100
	100	400	90	89	99.0
	75	300	90	88	98.0
	50	200	90	73	81.0
	25	100	90	30	33.2
Sodium crystalline penicillin in oil and beeswax (4.8 W/V)	200	800	50	48	96.0
	100	400	50	48	96.0
	75	300	50	46	92.0
	50	200	50	34	68.0
	25	100	50	11	22.0
Calcium penicillin in oil	200	800	50	37	74.0
	150	600	50	32	64.0
	100	400	50	26	52.0
	75	300	50	21	42.0
	50	200	50	2	4.0
	25	100	30	0	0.0
Controls	No treatment		60	0	0.0

TABLE II.
Protection with a Single Dose of Aluminum Penicillin in Oil.

Preparation	Units of penicillin	Survivors: No. of mice	Survivors: 8 days	Survivors: % protection
Aluminum penicillin in oil	400	50	50	100
	200	50	50	100
	150	50	46	92
	100	50	47	94
	75	50	45	90
Control	None	30	All dead 36 hrs	

fectively combats induced pneumococcus infection in mice. Compared with calcium penicillin in oil or sodium penicillin in peanut oil and beeswax preparation, the aluminum penicillin in oil is more effective on a unit for unit basis.

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